The role of the wingless gene in the control of growth and pattern formation during drosophila wing development

Thesis

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The role of the *wingless* gene in the control of growth and pattern formation during Drosophila wing development

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APPENDIX
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ABBREVIATIONS

AEL	after egg laying
A101/neu	*neuralized-lacZ*
arm	*armadillo*
as-c	*acheate scute-complex*
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumin
CIP	calf intestinal phosphatase
cDNA	complementary DNA
Ci	Curie
Ci	*Cubitus Interruptus*
DAB	3,3'-diaminobenzidine
dCTP	deoxycytidine triphosphate
ddH_2O	double distilled water
Dll	*Distal-less*
DMF	dimethylformamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dsh	*dishevelled*
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenbis(oxyethylnitilo)tetraacetic acid
FITC	fluorescein isothiocyanate
HEPES	hydroxyl piperazine ethanesulphonic acid

hr	hours
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase pairs of DNA
LB	Luria-Bertani broth
min	minutes
mRNA	messenger RNA
M	Molar
N	*Notch*
NGS	normal goat serum
NBT	nitro blue tetrazolium
OD	optical density
PBS	phosphate buffered saline
PEG	polyethylene glycol
rpm	revolutions per minute
sec	seconds
SMC	sensory mother cell
Sp	*Sternopleural*
spdfg	*spadeflag*
SSC	sodium chloride/sodium citrate (see Ausubel et al., 1994)
Su(H)	*Suppressor of Hairless*
TAE	tris-acetate buffer
tris	tris(hydroxymethyl)aminomethane
U	enzyme unit
vg	*vestigial*
v/v	volume for volume
w/v	weight for volume

wg	*wingless*
wt	wild-type

ABSTRACT

Recent work on Drosophila limb development has indicated that short-range interactions between distinctly specified populations of cells (compartments) establish organizing centers at compartment boundaries. These organizing centers direct pattern formation and growth in the developing limbs. In the Drosophila wing imaginal disc, there are at least two such organizing centers, located at the anterior/posterior (A/P) and dorsal/ventral (D/V) compartment boundaries. The genetic hierarchies which establish these organizers are starting to be understood, and it also appears that the key mediators of some of the organizers have been identified. Thus Decapentaplegic (Dpp, a secreted signalling molecule of the TGF-β family) is the mediator of the A/P organizer, while Wingless (DWnt-1, a secreted molecule of the Wnt family) is a key mediator of the D/V organizer.

In this thesis, several aspects of Wingless function in the wing imaginal disc are examined. Two regulatory mutations, *spade*^{flag} (*spdfg*) and *Sternopleural* (*Sp*), that affect Wingless expression in the wing imaginal disc are characterized. The analysis of the mutation *spdfg*, together with other data, identifies a role of Wingless as a localized mitogen in the developing wing hinge, and also indicates that cells in different regions of the wing disc respond very differently to the Wingless signal. The mutations *spdfg* and *Sp* are also among the tools used to examine the position of Wingless in the genetic hierarchy that establishes and mediates the activity of the D/V organizer. These experiments extend the evidence suggesting that Wingless mediates both short-range and long-range effects of the D/V organizer. Wg does so by controlling the expression domains of different target genes, including the *acheate-scute* genes, *Distal-less* and *vestigial*. Finally, the mechanism by which Wingless mediates the activity of the D/V organizer is examined. The results obtained suggest that Wingless functions as a long-range morphogen.

CHAPTER 1:
INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1: The Organizer concept

The spectacular transplantation experiments published by Spemann and Mangold in 1924 (reviewed in Hamburger, 1988) have had a very profound and lasting effect on developmental biology. When the dorsal blastopore lip from an early amphibian gastrula is transplanted into the ventral ectoderm of another gastrula, this group of transplanted cells is able to induce the formation of a whole new embryonic axis, made up to a large extent of host cells that would have normally formed ventral tissue. Spemann used the term organizer for the cells of the dorsal blastopore lip, because they were not only able to induce ventral cells to form dorsal tissue types, but also to organize the different tissue types into a correct D/V and A/P sequence. Organizers have been identified again and again by developmental biologists working on very different model organisms, indicating that pattern formation in multicellular organisms is controlled to a large extent by specialized groups of cells that influence the development of surrounding cells. A lot of effort has therefore been channeled into the elucidation of the mechanism of action of organizers. This problem can be broken down into two questions: a) how are organizers set up and b) how do they organize pattern in responding cells? These questions have shaped a large body of research in developmental biology, and with the advent of molecular biology, they have been adressed in terms of specific gene activities. These questions are also major themes of this thesis, which attempts to examine some of the roles played by the *wingless* gene in mediating organizer function during Drosophila wing development.

1.2: Morphogens in development

It is difficult to pin down the exact origin of the gradient morphogen theory. Gradient models for development have been around for many decades (for example, see Huxley and deBeer, 1934). In the early days of gradient theory, gradients were thought to be of metabolic nature (*eg.* Child, 1941), supported by the observation that such a metabolic gradient could actually be visualized by treating sea urchin embryos with a redox dye (Child, 1936; Horstadius, 1952). However, an alternative model, in which concentration gradients of specific substances could specify cell states in a concentration-dependent manner, was proposed already at the end of the thirties by Dalcq and Pasteels (Dalcq and Pasteels, 1937; Dalcq, 1938). Turing, among others, applied mathematical considerations to this model, and coined the term morphogen (Turing, 1952).

In 1969 Wolpert formulated the theory of positional information (Wolpert 1969,1989). This theory, best illustrated by the French Flag Model, states that position within a field of developing cells is specified by a coordinate system (defined by the distance from boundary regions), and that cells are able to respond to this positional value by differentiating in a particular way. This idea uncouples positional information from the final differentiation behaviour of cells. In other words, the same positional information could be used in several developmental contexts, the specificity in each case arising out of the competence of the responding cells. While this model does not absolutely require that a morphogen gradient must be used to generate positional information, this has remained the most attractive version of the model, especially after Crick (1970) published calculations suggesting that the existence of a diffusion gradient of a substance in an embryonic field (which are always small, see Wolpert, 1969) might be physically plausible.

Out of these considerations came the local source-dispersed sink (LSDS) model (reviewed in Slack, 1987, 1991), in which a locally produced morphogen diffuses away from its source and is degraded by responding cells, thereby forming a concentration gradient. The Spemann organizer, for example, could organize the embryonic axes by producing such a morphogen. A large body of work on the development of the chick limb suggested that the zone of polarizing activity (ZPA) may be the source of a morphogen (for example, see Tickle et al., 1975, 1976; Brickell and Tickle, 1989). Grafting experiments in insects were also interpreted as revealing the existence of a gradient of positional information that organizes pattern within insect compartments (Locke, 1959; Stumpf, 1966; Lawrence,1966).

A long list of candidate morphogens have been proposed to function in different contexts during development (see below). For many of these, the case is not proven and remains controversial, or is considered to be untypical of multicellular development (*eg.* Bicoid/Hunchback). However, in three cases, namely Activin in the amphibian gastrula, and Wingless and Dpp in the fly wing, the evidence appears to be rather convincing. If these cases are generally accepted, they should lend renewed plausibility to the morphogen model of positional information, and the emphasis of future research might be expected to lie not so much in proving the existence of morphogens, but in learning how they work.

1.2.1: Candidate morphogens

Retinoic acid

The finding that application of retinoic acid to the anterior chick limb bud mimicks the effect of a ZPA graft, and that retinoic acid induces different digits in a concentration-dependent manner (Tickle et al., 1982; Summerbell, 1983; Tickle et al., 1985), led to the proposal that retinoic acid is the

morphogen that mediates the activity of the ZPA (reviewed in Tickle 1991; Tickle & Eichele 1994). However, it has recently been shown that retinoic acid induces the expression of Sonic hedgehog, which is normally expressed in the ZPA; furthermore, expression of Sonic hedgehog anteriorly is also sufficient to mimic the effect of the ZPA (Riddle et al., 1993), suggesting that retinoic acid acts indirectly, by inducing Sonic hedgehog expression. This raises an important point. A demonstration that a substance has a concentration-dependent effect on pattern formation, while consistent with it being a morphogen, does not prove the case. Any factor which activates a morphogen is expected to have a concentration-dependent effect. It is therefore necessary to show that a locally produced factor acts not only in a concentration-dependent manner, but also directly at a distance on responding cells.

Activin

It has been shown that activin can specify different types of mesoderm in Xenopus animal pole cells in a concentration dependent manner, and that the sequence of concentration thresholds corresponds to the sequence in which these mesodermal cell types are normally found in the D/V axis of the embryo (Green & Smith 1990, 1992). These studies were extended by Gurdon and colleagues (Gurdon et al., 1994, 1995), who reproduced the experiments in solid tissue and also showed that the effect of activin can be achieved with purified activin protein, that long-range effects can be achieved in the absence of cell division, and that activin can signal across an intervening layer of cells in which protein synthesis is inhibited. Also, Jones et al. (1996) have shown that constitutively active forms of two activin receptors turn on activin target genes in a strictly cell autonomous manner, indicating that activin does not initiate a signal relay mechanism.

Taken together, these results strongly indicate that activin acts directly at a distance, and turns on different target genes in a concentration-dependent manner. This makes it tempting to speculate that an activin-like gene is produced by the cells of the Nieuwkoop center, and functions as a morphogen in inducing different mesodermal cell types, which is supported by the observation that a dominant negative activin receptor abolishes all mesoderm formation (Hemmati-Brivanlou and Melton, 1993; Wittbrodt and Rosa, 1995). However, it is still necessary to show that an activin-like factor is expressed *in vivo* in the right place and the right time, and more importantly, that it is required for mesoderm induction and patterning.

The model for activin as a morphogen has also been complicated by several studies showing that the concentration-dependent effects of activin on cell phenotype are not immediately achieved in the responding cells, but instead arise after many hours and require communication among the responding cells (Wilson and Melton, 1994; Green et al., 1994; Symes et al., 1994; reviewed in Cooke, 1995). This is interesting, because it addresses the issue of how directly a morphogen can specify an ultimate pattern. These results suggest that activin functions rather indirectly, perhaps by specifying a relatively crude prepattern, which is subsequently refined, or focussed, by other cell-cell interactions.

Jones et al. (1996) also show that two other TGF-β family members, Xnr-2 and BMP-4, activate the same target genes as activin, but, in contrast to activin, only over a very short range. This appears to be due to a lower efficiency in the processing and secretion of Xnr-2, as well as the presence of the extracellular matrix (Jones et al., 1996). These results suggest that not all secreted signalling proteins have long-range effects, and that the regulation of the amount of functional signal which is secreted, as well as interactions with the extracellular matrix, can regulate the range of a signalling factor.

Sonic hedgehog

Sonic hedgehog is expressed by cells of the vertebrate notochord and floorplate and has been shown to mediate the floor plate- and motor neuron inducing activities of these tissues (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994; Chiang et al., 1996). While the floor plate is induced close to the the notochord, *ie.* the source of the signal, motor neurons are induced a few cells further away. (Roelink et al. 1995) have presented data showing that low concentrations of Sonic hedgehog induce motor neurons in the absence of floor plate, while high concentrations of Sonic hedgehog induce floor plate at the expense of motor neurons. This suggests that Sonic hedgehog, secreted by the notochord, may function as a morphogen to pattern the ventral neural tube. However, it still remains to be shown that this effect is direct and at a distance, especially since floor plate cells themselves exhibit the same inductive abilities as notochord (*eg.* Placzek et al., 1993). While it has been shown that antibodies against Sonic hedgehog inhibit the induction of motor neurons by the notochord (Marti et al., 1995), even at very late stages (Ericson et al., 1996), this still does not exclude the possibility that Sonic hedgehog activates an intermediary factor, which then mediates motor neuron induction.

Drosophila hedgehog

hedgehog is expressed in a narrow stripe of cells at the parasegmental compartment boundary of the Drosophila embryo (Lee et al., 1992; Mohler & Vani, 1992; Tabata et al., 1992). Heemskerk and DiNardo (1994) have presented data suggesting that Hedgehog patterns the segment in a concentration-dependent manner. The denticles secreted by the dorsal epidermis of the Drosophila larva are arranged in rows of different types that have a specific sequence with respect to the compartment boundary (*ie.* the source of Hedgehog). Heemskerk and DiNardo show that expressing Hedgehog ubiquitously at different levels specifies different

denticle types ubiquitously, with the type of denticle specified at increasing levels of Hedgehog corresponding to those which are normally found closer to the source of Hedgehog protein. Again, this is consistent with Hedgehog being a morphogen, but does not prove the case, as Hedgehog could be acting indirectly. For example, Hedgehog also has long range effects in the imaginal discs, but it has been shown that these are mediated by Dpp and Wingless, which are activated in response to Hedgehog (Basler & Struhl, 1994; Zecca et al., 1995)

Ingham and Fietz (1995) have addressed this issue by elevating the amount of Hedgehog produced within its normal domain of expression. If Hegehog were a morphogen, this should perturb the Hedgehog gradient by increasing the level at the source, and should result in the expansion of fates normally found close to the Hedgehog-expressing cells. This result was not observed, suggesting that Hegehog may not function as a morphogen, at least in this context.

Decapentaplegic

Dpp has been proposed to function as a morphogen both in the Drosophila embryo (Ferguson and Anderson, 1992) and in the wing imaginal disc (Lecuit et al., 1996; Nellen et al., 1996). In the embryo, injection of different amounts of *dpp* RNA have a concentration-dependent effect on D/V patterning, the highest concentrations giving the most dorsal structures. This is consistent with the observation that Dpp is normally expressed in the dorsal third of the embryo, and is required for the formation of dorsal structures (Irish & Gelbart, 1987; St. Johnston & Gelbart, 1987). However, it has not been shown that this concentration-dependent effect is direct, and the results presented by Ferguson and Anderson can only be achieved in *snake-Toll*^{D} embryos, which have a lateralized phenotype. Dpp does not have an effect on ventral cells in wild-type embryos, probably because these cells express the *sog* gene, which encodes a secreted factor that has recently

been shown to bind and inhibit Dpp (Holley et al., 1996; Piccolo et al., 1996). The inability of Dpp to induce dorsal fates in ventral cells is difficult to reconcile with its proposed role as an instructive morphogen in the embryo.

In the wing, the case is better. Nellen et al. (1996) and Lecuit et al. (1996) show that the genes *spalt* and *omb* are expressed in nested domains centered on the domain of Dpp expression at the A/P compartment boundary. By misexpressing an activated form of the Dpp receptor, it is shown that *spalt* and *omb* are direct targets of the Dpp pathway, since these genes are only activated in cells expressing the activated receptor, but not in surrounding cells. Consistent with this observation, the activity of the Dpp signal transduction pathway is required autonomously for the expression of *spalt* and *omb* in cells located far from the source of Dpp. These experiments indicate that Dpp acts directly and at a distance. It is also shown that Dpp has a concentration-dependent effect, as elevating the level of Dpp expression within its normal domain (with dppGAL4:UASdpp), causes a broadening of the domain of Spalt expression and overgrowth of the wing disc. This indicates that the amount of Dpp secreted by cells at the A/P boundary directly defines the extent of target gene expression and controls the size of the wing pouch. Singer et al. (1997) also show that cells which are reduced in their ability to receive the Dpp signal autonomously assume a fate that is normally found further away from the source of Dpp. These are exactly the properties expected of a morphogen. Both *spalt* and *omb* have critical functions in wing development (de Celis et al., 1996b; Grimm & Pflugfelder, 1996; Lecuit et al., 1996; Sturtevant et al., 1997), and are therefore mediators of long-range signalling by Dpp.

Wingless

Like Dpp, Wingless has been proposed to be a morphogen in several developmental contexts, namely the leg imaginal disc (Struhl & Basler, 1993), the wing imaginal disc (Zecca et al., 1996; Neumann & Cohen, 1997; see Chapter 6 of this thesis), the embryonic midgut (Hoppler & Bienz, 1995), and in the embryonic parasegment (Bejsovec & Martinez-Arias, 1991; Lawrence & Struhl 1996). Again, like Dpp, the case can only be considered solid in the wing disc.

Bejsovec and Martinez-Arias (1991) already noted a concentration-dependent effect of Wingless in the embryo, and as they could detect Wingless up to four cells away from its source, proposed that Wingless might pattern cells directly within that range. Lawrence et al. (1996) show that ubiquitous expression of different levels of Wingless in a *wingless*$^-$ *engrailed*$^-$ background specifies different denticle types (similar to Hedgehog, see above), but cannot distinguish between a direct or indirect effect. The only known target of Wingless that mediates patterning of the embryonic parasegment is *engrailed*, which has been shown to be activated in response to Wingless only in immediately adjacent cells (Vincent & Lawrence, 1994).

In the midgut, Wingless, expressed in the visceral mesoderm of parasegment 8, is required for the differentiation of two different cell types in the nearby endoderm: 'large flat cells' require high levels of Wingless and develop immediately adjacent to the source of Wingless, while 'copper cells' require lower levels of Wingless and are repressed at high levels, therefore developing several cells away from the source of Wingless (Hoppler and Bienz, 1995). This is reminiscent of the concentration-dependent specification of motor neurons and floor plate by Sonic hedgehog (see above). In both cases, either response can be triggered in the absence of the other response, depending only on the level of signal provided. However, in neither case has it been shown yet that this effect is direct. The homeotic gene *labial* is expressed in a graded manner in

the Wingless-dependent copper cell domain, with highest levels close to the source of Wingless (Hoppler and Bienz, 1995), consistent with the proposed concentration-dependent effect of Wingless.

In the leg imaginal discs, Wingless is expressed in a ventral wedge and is required for the formation of ventral fates (Baker 1988a; Couso et al. 1993). Struhl and Basler (1993) showed that expressing low levels of Wingless ectopically is sufficient to specify ventrolateral fates, but not the most ventral fates. It has since been shown that a high level of Wingless expression is able to fully ventralize dorsal cells (Brook & Cohen 1996; Jiang & Struhl 1996), suggesting that Wingless functions in a concentration-dependent manner to specify ventral cell fates. However, it has not been shown that this is a direct effect. The gene *H15* is expressed in a broad domain centered on the domain of Wingless expression in the leg, and is activated cell-autonomously in *shaggy/zeste-white3* mutant clones (inactivation of *shaggy/zeste-white3* is equivalent to activation of the wingless pathway; see Siegfried et al., 1992), indicating that *H15* is a direct target of Wingless signalling in the leg (W. Brook, unpublished result), and providing evidence that Wingless signals directly and at a distance in the leg disc. However, an *H15* null mutant has no leg phenotype (Brook and Cohen, 1996), indicating that *H15* is not essential for the mediation of Wingless signalling. Since no other targets for Wingless in the D/V axis of the leg disc are known, this makes it difficult to prove that Wingless functions as a morphogen in patterning this axis.

The best evidence for Wingless as a morphogen comes from work on the wing imaginal disc. The experiments done as part of this thesis will be presented and discussed in Chapter 6 (see also Neumann and Cohen, 1997). Very similar experiments were independently conducted in the laboratories of K. Basler and G. Struhl (Zecca et al., 1996), and these authors reach practically identical conclusions. These studies will also be discussed in Chapter 6.

Vertebrate Wnts in embryonic D/V axis determination

A large family of vertebrate Wnts has been identified, and members of this family appear to function in many different developmental contexts (reviewed in Parr and McMahon, 1994). One of the most studied effects of Wnt signalling on vertebrate development is D/V axis formation in the early embryo. Ectopic expression of Wnt-1 and several related members of the Wnt family lead to a duplication of the primary embryonic axis (McMahon & Moon, 1989; Smith & Harland, 1991; Sokol et al., 1991). It has also been shown that depletion of maternal β-catenin (which is required to transduce the Wnt signal) with antisense oligonucleotides suppresses the formation of dorsal structures (Heasman et al., 1994). This suggests that the activation of the Wnt signalling pathway is not only sufficient, but also necessary for the establishment of a D/V axis.

The induction of a whole embryonic axis is a long-range effect, and it is tempting to speculate that a vertebrate Wnt might function as a long-range morphogen in specifying dorsal structures. However, several results argue against this model. Firstly, a membrane-tethered form of Wnt-1, which is unable to diffuse away from the cells in which it is produced, is able to non-autonomously induce an ectopic D/V axis (Parkin et al., 1993), suggesting that the long-range effect of Wnt-1 is indirect in this context. Also, an embryo which has been depleted of its maternal supply of β-catenin can be rescued by the injection of β-catenin mRNA into just one of the blastomeres of a 32-cell stage embryo, (Wylie et al., 1996). Again, this is a non-autonomous effect on un-injected cells and suggests that the Wnt signalling pathway activates a second signal, which relays the long range effect. Consistent with this proposal, the Wnt signalling pathway appears to function upstream of all other genes known to affect axis formation in the Xenopus embryo, including Siamois (Carnac et al., 1996; Wylie et al., 1996). Siamois is also able to induce a complete secondary axis, and appears to be expressed in the dorsal

endoderm, which corresponds to the Nieuwkoop center (Lemaire et al., 1995). Taken together, these results suggest that local activation of the Wnt signalling pathway on the dorsal side of the early Xenopus embryo leads to the establishment of a Nieuwkoop center, which then serves as the source of a long-range morphogen (perhaps an activin-like molecule) that patterns the D/V axis.

It is interesting to note that in the Drosophila wing imaginal disc, Wingless (which also triggers axis duplication in Xenopus, see Chakrabarti et al., 1992) appears to act directly at a distance and as a morphogen (Zecca et al., 1996; Neumann and Cohen, 1997; see also Chapter 6 of this thesis), while in D/V axis establishment in the Xenopus embryo, the Wnt signalling pathway appears to act indirectly, by initiating a relay signal (Carnac et al., 1996; Wylie et al., 1996). This suggests that the same signal can be used as a morphogen in some contexts, and as a short-range inducer in others.

It should be pointed out that D/V axis initiation in Xenopus, while requiring the activity of the Wnt signal transduction pathway, may not require a Wnt ligand. Overexpression of a dominant-negative form of Wnt-8 suppresses the formation of an ectopic D/V axis by Wnt-1 and related Wnts, but does not suppress endogenous axis formation (Hoppler et al., 1996). One possible explanation for this result is that cortical rotation, which triggers the formation of the Nieuwkoop center dorsally (reviewed in Gerhart et al., 1989), may activate the Wnt signal transduction cascade in the absence of a Wnt ligand.

Bicoid and Hunchback

Bicoid was the first morphogen to be proposed for Drosophila (reviewed in St. Johnston & Nüsslein-Volhard 1992). Bicoid is both necessary and sufficient to induce anterior structures in the embryo, and the site at which Bicoid protein is produced determines the A/P polarity of these structures (Driever & Nusslein-Volhard, 1988a,b). Bicoid RNA

is localized to the anterior pole of the embryo, and the translated protein forms a diffusion gradient from this source (Driever & Nusslein-Volhard, 1988a). Increasing the dosage of Bicoid increases the amount of anterior structures at the expense of posterior structures, and shifts them further posteriorly (Driever & Nusslein-Volhard, 1988b). All these features are consistent with Bicoid functioning as a morphogen. However, Hunchback, a target of Bicoid, has properties of a morphogen itself (Struhl et al., 1992), and it has recently been shown that Hunchback is absolutely required for all Bicoid-dependent A/P patterning (Simpson-Brose et al., 1994). This suggests that Bicoid and Hunchback cooperatively control A/P patterning. This is is feasible because both proteins are transcription factors that are free to diffuse through the nuclear syncitium of the Drosophila embryo, a feature which also makes the generality of this kind of morphogen doubtful.

Spaetzle/Dorsal

While A/P patterning of the Drosophila embryo is controlled by transcription factor morphogens diffusing among the nuclei of the embryonic syncitium, D/V patterning, and patterning of the terminal regions is controlled by signals produced outside the embryo (reviewed in St. Johnston and Nuesslein-Volhard, 1992; Chasan and Anderson, 1993). A cascade of proteases acting in the periviteline space appears to be activated only on the ventral side of the embryo, leading to the proteolytic activation of Spaetzle, the proposed ligand for Toll, in this region (Morisato and Anderson, 1994). Activation of the Toll receptor is transduced into the embryo and directs a D/V gradient of nuclear localization of the Dorsal transcription factor (Roth et al., 1989; Rushlow et al., 1989; Steward et al., 1989). The nuclear concentration of Dorsal then controls several target genes in different spatial domains along the D/V axis by binding directly to their enhancer sequences with different affinities, and by activating some targets, while repressing others (Thisse et al., 1991; Jiang et al., 1991; Pan et

al., 1991). While these data make a good case for a morphogenetic gradient of Dorsal protein directing D/V patterning in the embryo, it is not exactly clear how this gradient is achieved. A gradient in the activation of the proteolytic cascade in the extracellular space could lead to a gradient of activated Spaetzle. Alternatively, Spaetzle, activated at the ventral midline, could diffuse away from its source, thereby forming a gradient. Also, the Toll receptor transduces the Spaetzle signal into a syncitium, and here components of the signal transduction machinery, including Dorsal, are free to diffuse. So, while it appears fairly clear how graded information is translated into discrete cell states in this system, it is not clear at which level this graded information is generated in the first place.

Spitz

A set of genes collectively termed the *spitz* group are required to pattern the ventrolateral region of the Drosophila embryo after gastrulation (Mayer & Nusslein-Volhard, 1988). In contrast, the Toll/Dorsal pathway and the Dpp pathway are required for D/V patterning before gastrulation. The *spitz* group includes both the Drosophila EGF receptor (DER), and *spitz* itself, which appears to encode a ligand for DER (Schweitzer et al., 1995). The DER pathway is required for the establishment of ventral fates (Raz and Shilo, 1993), and recent evidence has suggested that Spitz, which is produced as an inactive trans-membrane protein, is cleaved and activated only in the cells of the ventral midline, leading to the proposal that it diffuses away from its source and thereby forms a concentration gradient (Golembo et al., 1996). Consistent with this proposal, high levels of Spitz lead to a stronger activation of MAPK *in vitro* than lower levels, suggesting that the level of activity of the DER signalling pathway depends on the local concentration of Spitz (Schweitzer et al., 1995).

Several target genes for DER signalling have recently been identified, including *ventral nervous system defective* and *pointed P1* (Gabay et al., 1996). These genes are activated in nested domains centered on the source of Spitz at the ventral midline, consistent with a concentration-dependent effect of Spitz on different targets. Once again, however, it is not clear whether these genes are direct targets of the DER pathway, and whether Spitz acts directly at distance.

In conclusion, a lot of evidence implicates different molecules as morphogens, both in Drosophila and in vertebrates. While many of these have not been proven to be morphogens, the favourable case for some makes the case for others more plausible. The implication is that morphogen gradients may be a very general mechanism to generate positional information in animal development. The fact that similar molecules (*eg.* members of the TGF-β, Wnt, and Hedgehog families of secreted signalling proteins) are implicated as morphogens in many different contexts, not only in the same animal, but also in animals as remotely related as flies and vertebrates, would seem to point towards the universality of positional information proposed by Wolpert (1969). On the other hand, morphogen gradients are clearly not the only way to pattern multicellular organisms. For example, pattern formation in the Drosophila eye appears to be controlled almost exclusively by short-range cell interactions (Tomlinson, 1988; Freeman, 1997).

1.3: Compartments and boundaries control Drosophila development

1.3.1: Establishing compartments

Work done by A. Garcia-Bellido and colleagues has shown that Drosophila is subdivided into distinct populations of cells, termed compartments (García-Bellido et al., 1973, 1976; García-Bellido, 1975). Compartments were first recognized because of the boundaries that run between them. These are straight lines which cells never cross, even if given a growth advantage. In other words, the descendants of one cell only contribute to the compartment of their origin. Garcia-Bellido proposed that compartment-specific identiy is controlled by the expression of selector genes in the founder cells of a particular compartment. This proposal has been supported by a large body of data; for example, the homeobox gene *engrailed* has been shown to be the selector gene for a subset of compartments (reviewed in Lawrence & Morata, 1994).

The subdivision of the adult structures into compartments is already initiated in the embryo. The cascade of transcription factors that is triggered by the localized determinant Bicoid culminates in the subdivision of the embryo into a series of repeating units along the A/P axis called parasegments (Martinez Arias & Lawrence, 1985). Each parasegment is divided into an anterior group of cells that expresses Engrailed protein, and a posterior group that does not. The primordia of the thoracic imaginal discs (which give rise to thoracic adult structures during larval and pupal development) arise straddling the parasegment boundaries and hence consist of two cell populations, the posterior of which expresses Engrailed (Cohen, 1990; Cohen et al., 1993; reviewed in Cohen, 1993). Cell lineage analysis has shown that the boundary between the Engrailed expressing- and non expressing cells already functions as a lineage restriction boundary in the embryo (Steiner, 1976; Wieschaus & Gehring,

1976). In other words, the imaginal discs inherit their A/P compartmentalization from the embryo. It has recently been shown that the homeodomain gene *engrailed* does not function alone, but together with its homologue *invected,* to specify posterior compartment identity (Guillen et al., 1995; Sanicola et al., 1995; Simmonds et al., 1995; Tabata et al., 1995).

While the A/P subdivision of the wing imaginal disc is derived from the earlier subdivision of the embryonic ectoderm, a second subdivision during larval development divides it into a dorsal and a ventral compartment (Garcia-Bellido, 1973). The dorsal compartment is specified by the localized expression of the LIM/homeodomain selector gene *apterous* (Diaz-Benjumea & Cohen, 1993; Blair et al., 1994). However, it is not known how the localized expression of *apterous* is controlled. While there appears to be a D/V subdivision of the leg imaginal disc (Steiner 1976), this is not due to the localized expression of a selector gene, and hence does not represent a lineage restriction (Brook and Cohen, 1996; Jiang and Struhl, 1996). There is also evidence that the wing disc is divided into a proximal and a distal compartment, roughly at the same time as the D/V compartmentalization (Garcia-Bellido et al., 1973), but little more is known about this event.

1.3.2: Short-range interactions between compartments establish organizing centers at compartment boundaries

Cells in the posterior compartment express the secreted signalling protein Hedgehog under the control of Engrailed (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992). Hedgehog diffuses to nearby anterior cells, and there activates the expression of Dpp (Basler and Struhl, 1994; Tabata & Kornberg, 1994). Posterior cells are unable to respond to Hedgehog because they express Engrailed and Invected

(Sanicola et al., 1995; Tabata et al., 1995; Zecca et al., 1995). While ectopic expression of Hedgehog only causes repatterning in the anterior compartment, ectopic expression of Dpp in either compartment is sufficient to organize an ectopic A/P axis, centered on the source of Dpp (Zecca et al., 1995). Thus the role of the A/P compartment boundary is to provide a localized source of Hedgehog for anterior cells, which respond by activating Dpp, which in turn organizes pattern and growth symmetrically in both compartments (reviewed in Blair, 1995; Lawrence & Struhl, 1996; Brook et al., 1996).

Similarly, it has been shown that the D/V compartment boundary of the wing, located at the *apterous*$^{+}$/*apterous*$^{-}$ interface, serves as an organizing center that specifies pattern in the D/V axis and controls growth (Diaz-Benjumea and Cohen, 1993). As in the case of the A/P organizer, ectopic juxtaposition of dorsal and ventral cells leads to the formation of a secondary axis, for example if a clone of cells lacking *apterous* function is generated in the dorsal compartment (Diaz-Benjumea and Cohen, 1993; Blair et al., 1994; Williams et al., 1994). The D/V compartment boundary corresponds to the wing margin in the adult wing, and an ectopic wing margin is generated at an ectopic *apterous*$^{+}$/*apterous*$^{-}$ interface. At the same time, surrounding wild-type cells are induced to overproliferate, forming an outgrowth from the surface of the wing blade. Thus both the A/P and D/V boundaries have organizing properties which are analogous to those of the Spemann organizer.

Apterous induces the expression of Fringe, a putative secreted protein, in dorsal cells (Irvine & Wieschaus, 1994). Fringe, in turn, induces the expression of *Serrate*, and also makes dorsal cells unresponsive to Serrate (Kim et al., 1995). *Serrate* encodes a transmembrane ligand for the Notch receptor (Rebay et al., 1991), which signals to nearby ventral cells to activate *vestigial* as well as *wingless* in parallel (Kim et al., 1995, 1996; Rulifson & Blair, 1995; Diaz-Benjumea & Cohen, 1995; Couso et al., 1995; de Celis et al., 1996a; Doherty et al.,

1996; Neumann & Cohen, 1996b; see also Chapter 5 of this thesis). In the case of the D/V organizer, a second signal is sent from ventral to dorsal cells, leading to the symmetric expression of *vestigial* and *wingless* Straddling the D/V boundary (Blair, 1993; Williams et al., 1994; Diaz-Benjumea and Cohen, 1995). This second signal may be mediated by Delta (DeCelis et al., 1996a; Doherty et al., 1996), another trans-membrane ligand of Notch (Rebay et al., 1991; Fehon et al., 1990).

Activity of *Notch* is critical for wing development (Schellenbarger & Mohler, 1978) and is required in cells abutting the D/V boundary (de Celis & Garcia Bellido, 1994). Clones of cells lacking *Notch* activity that touch the D/V boundary from one side, not only lose expression of *wingless* autonomously on that side, but also cause loss of *wingless* expression in wild-type cells on the other side of the D/V boundary (Rulifson and Blair, 1995). This suggests that a Notch-dependent feedback loop maintains the activated state of Notch on both sides of the D/V boundary, perhaps through the regulation of *Serrate* and *Delta* expression (Rulifson and Blair, 1995; Diaz-Benjumea and Cohen, 1995; Kim et al., 1995; DeCelis et al., 1996a; Doherty et al., 1996). Experiments using a hypomorphic Notch allele show a greater requirement for Notch on the ventral side of the D/V boundary (DeCelis et al., 1996a). Taken together with the observation that *wingless* appears to be activated first on the ventral side (Ng et al., 1996), this suggests that the first signal triggering the formation of the D/V organizer is a dorsal to ventral one.

1.3.3: Long-range signalling events mediate the activity of boundary organizers

As already mentioned in the previous section, ectopic expression of Dpp is able to trigger an extra A/P axis, indicating that Dpp is not only necessary, but also sufficient to

mediate the activity of the A/P organizer in the wing. Also, as already discussed in section 1.2.1 above, Dpp appears to exert this effect by functioning as a morphogen. Thus the A/P organizer serves as a localized source of a secreted signal that then acts directly at a distance to control pattern formation, growth and polarity in the A/P axis. Likewise, Wingless is not only necessary for the activity of the D/V organizer, but ectopic expression of Wingless is also able to mimic the effect of an ectopic D/V interface (Blair, 1992, 1994; Phillips & Whittle, 1993; Couso et al., 1994; Diaz-Benjumea and Cohen, 1995; Neumann and Cohen, 1996b, 1997; Zecca et al., 1996; see also Chapters 5 and 6 of this thesis). Also like Dpp, Wingless appears to act directly at a distance, and in a concentration-dependent manner to control patterning and growth in the wing (Zecca et al., 1996; Neumann and Cohen, 1997; see also Chapter 6 of this thesis).

As noted above in section 1.2.1, Wingless has also been implicated as a morphogen in mediating the organizing activity of other compartment boundary organizers, for example the ventral A/P compartment boundary of the leg (Struhl and Basler, 1993), or the parasegment boundary of the embryo (Bejsovec and Martinez-Arias, 1991; Lawrence et al., 1996). Hedgehog, expressed on the other side of the embryonic parasegment boundary from Wingless, has also been proposed to be a morphogen (Heemskerk and DiNardo, 1992). Taken together, these results suggest that morphogens, expressed at compartment boundaries, may function in general to mediate organizer activity.

In conclusion, it would appear that the answers to the two questions: first, how organizers are established, and second, how they function, are starting to become tangible in Drosophila. Short-range interactions between differently specified populations of cells induce the organizer at a compartment boundary. Then, long-range signalling by a morphogen mediates the function of this organizer.

Of course, this immediately raises the question whether the same answers will also hold true for other animals, such as vertebrates. To date, there is little evidence for the existence of compartments in vertebrates. However, cells do become committed to different germlayers during gastrulation (for example, see Ho and Kimmel, 1993). Short-range inductions between two germlayers could serve to establish an organizing center, for example as in the case of floor plate induction by the notochord.

Also, there is evidence in Drosophila that lineage restriction is not the only way to generate a stable boundary between two cell populations. The expression domains of Dpp at the dorsal A/P compartment boundary of the leg and of Wingless at the ventral A/P compartment boundary of the leg imaginal disc are both induced by the Hedgehog signal (Basler and Struhl, 1994), and mutual repression by Dpp and Wingless is sufficient to maintain this state stably (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton & Hoffmann, 1996). There is also evidence that this interface between Dpp-expressing and Wingless-expressing cells is used to generate a proximal/distal (P/D) organizing center in the leg (Campbell et al., 1993; Campbell & Tomlinson, 1995; Diaz-Benjumea et al., 1994). Thus it is possible that short-range interactions between different groups of cells that are not specified by selector genes may also be used in vertebrates to establish organizing centers.

Evidence suggesting that morphogens may also be used in vertebrates to mediate organizer function has already been discussed in section 1.2.1 above. It may therefore be that similar principles apply both to the establishment and the mediation of organizer function in vertebrates and invertebrates.

1.4: Wing imaginal disc development in Drosophila

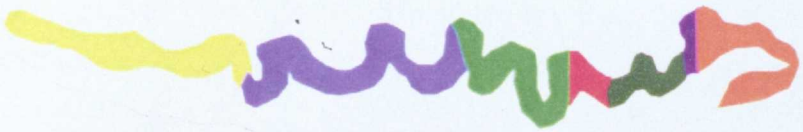
The thoracic imaginal discs are specified as small groups of cells that invaginate from the embryonic ectoderm (reviewed in Cohen, 1993). These imaginal discs arise straddling the parasegment boundary in the three thoracic segments, due to the combined action of Wingless and Dpp, which is required for the activation of Distal-less in these primordia (Cohen, 1990; Cohen et al., 1991, 1993). Later on, the wing and haltere discs arise by separating from the leg disc primordia in the second and third thoracic segments, respectively, and migrate dorsal-wards (Cohen et al., 1993). These groups of cells can be distinguished due to their expression of Vestigial.

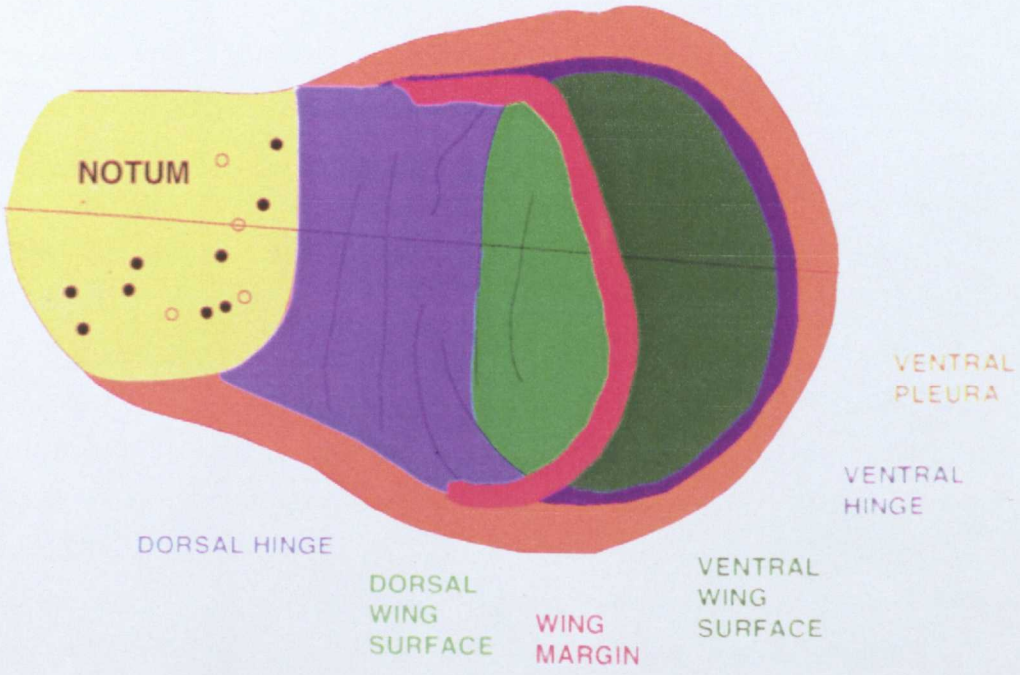
When the wing disc primordium is first established, it consists of less that fifty cells (García-Bellido & Merriam, 1971; Ripoll, 1972; Lawrence & Morata, 1977; Bate & Martinez-Arias, 1991). In contrast, the mature wing disc consists of about fifty thousand cells (Garcia-Bellido and Merriam, 1971). Proliferation in the wing disc occurs throughout larval development, and is not localized to any specific regions of the disc, occurring at a similar rate throughout the disc (González-Gaitán et al., 1994).

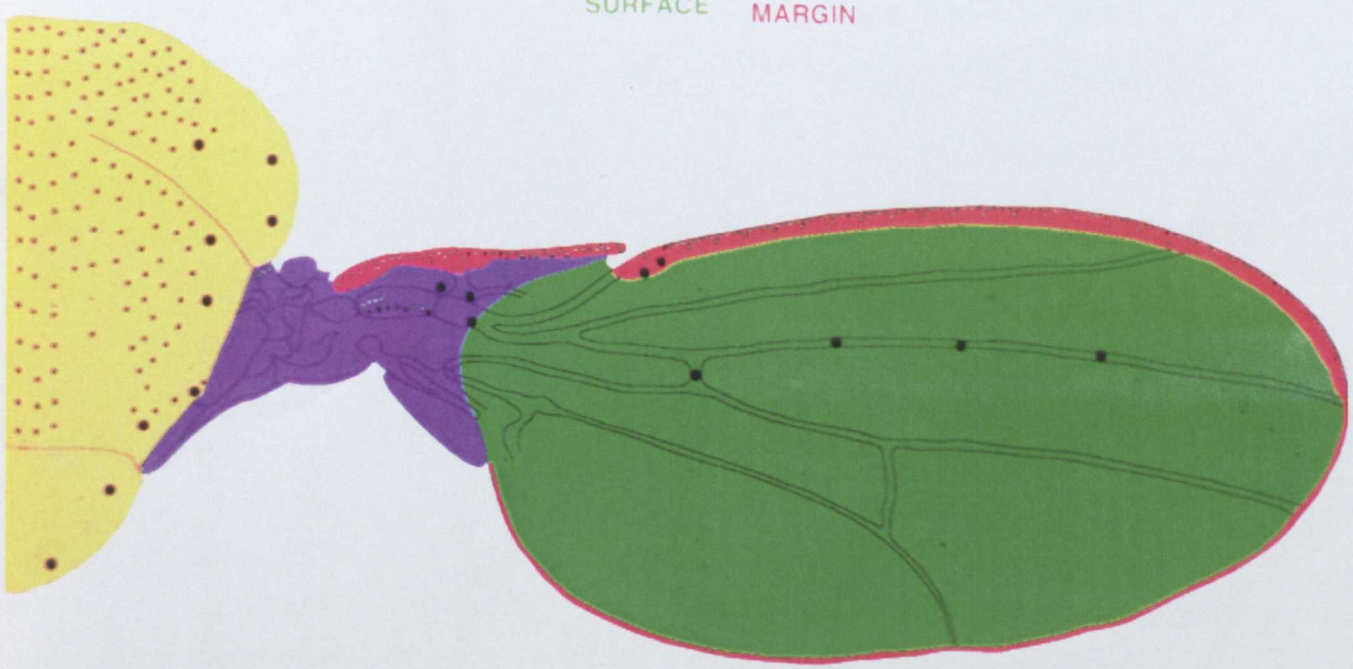
At the end of larval development, the wing imaginal disc undergoes extensive refolding before secreting cuticle and giving rise to the adult wing and thoracic body wall (reviewed in Fristrom & Fristrom, 1993). The cells located at the D/V boundary of the disc (which will give rise to the wing margin) move upwards out of the plane of the disc epithelium, and pull the two adjacent sheets of cells (the dorsal and ventral wing pouch) up with them. These two sheets thus become juxtaposed, leading to the generation of an adult wing that consists of two layers, a dorsal and a ventral one. These rearrangements are easiest to visualize by comparing the fate map of the mature wing disc with the cooresponding structures in the adult wing (Fig. 1.4.1).

Figure 1.4.1: Fate map of the Drosophila wing imaginal disc.

Regions of the wing disc that correspond to adult structures are coloured similarly and labelled. Note that during eversion, the refolding events cause the cells at the prospective wing margin to move upwards out of the epithelium, pulling the adjacent sheets of cells after them, thus causing these two sheets (the prospective dorsal and ventral surface of the wing) to become juxtaposed. This transforms a single-layered epithelium in the disc to a double-layered one in the adult wing, only the dorsal surface of which is shown in the lower panel of the diagram.




NOTUM
VENTRAL PLEURA
VENTRAL HINGE
DORSAL HINGE
DORSAL WING SURFACE
WING MARGIN
VENTRAL WING SURFACE



1.5: Wingless (DWnt-1) has multiple functions during Drosophila development

1.5.1: Functions of Wingless during embryonic development

The first *wingless* mutation to be identified (wg^1) leads to the loss of the wing and to duplication of the notum (Sharma & Chopra, 1976). Subsequently, *wingless* was found among the embryonic lethal mutations identified in the screen of Nüsslein-Volhard & Wieschaus (1980), and fell into the class of genes that give a segment polarity phenotype when mutated, which also include *engrailed.*

In the embryo, Wingless is expressed in narrow stripes of cells that are immediately anterior to the parasegment boundary, while Engrailed is expressed immediately posterior to this boundary (Baker, 1987; Martinez Arias et al., 1988; DiNardo et al., 1988). The expression of both Wingless and Engrailed are initiated by pair-rule genes (Ingham & Martinez-Arias, 1992), but subsequently come to be mutually dependent (Martinez-Arias et al., 1988; DiNardo et al., 1988; Heemskerk et al., 1991; Bejsovec and Martinez-Arias, 1991). Thus one of the early functions of Wingless is to maintain Engrailed expression, which is in turn required for the stability of the parasegment boundary. However, this is not the only contribution of Wingless to the patterning of the ectoderm. In the absence of *wingless* function, the whole embryonic ectoderm is covered by denticles (Bejsovec and Martinez-Arias, 1991; Bejsovec & Wieschaus, 1993). Ubiquitous expression of Wingless (generated by a hs-*wingless* transgene) leads to the opposite phenotype, in which the ectoderm is completely naked (Noordermeer et al., 1992). These experiments suggest that Wingless specifies the naked cuticle which is found straddling the parasegment boundary. Dougan & DiNardo (1992) have also shown that Wingless promotes two different cell types among Engrailed-expressing cells. It has

recently been proposed that Wingless functions as a morphogen in patterning the embryonic ectoderm (Lawrence et al., 1996).

The earliest function of Wingless in the embryo is a requirement for the specification of several types of neuroblasts in the neurectoderm (Chu-Lagraff and Doe, 1993). Wingless is required for the expression of the genes *even skipped* and *runt* in these cells, which are located close to the Wingless stripes in the ectoderm.

Wingless also mediates a signalling event between the mesoderm and the endoderm. As mentioned previously, Wingless, expressed in parasegment 8 of the visceral mesoderm, is required for cell-fate specification and for the expression of *labial* in nearby endodermal cells (Bienz, 1994; Immergluck et al., 1990; Reuter et al. 1990). A few hours later, Wingless activity is required for the formation of heart precursors in the dorsal-most mesoderm (Wu et al., 1995; Park et al., 1996).

Wnt-1, the vertebrate orthologue of Wingless, was first identified as a proto-oncogene (Nusse & Varmus, 1992). It is therefore interesting that a role for Wingless in the regulation of cell proliferation has been found in the anlage of the Malphigian tubules of the Drosophila embryo (Skaer & Martinez Arias, 1992). Wingless is expressed in the anlage and later at the tips of the Malphigian tubules. While loss of *wingless* activity leads to underproliferation in these strucures, ectopic expression using the hs-*wingless* leads to overproliferation, suggesting that Wingless stimulates cell division in the Malphigian tubules. A stimulatory effect of Wingless on proliferation of specific cell populations in the wing imaginal disc will be presented in chapters 5 and 6 of this thesis.

Finally, Wingless also has a function in the specification of thoracic imaginal disc primordia in the embryonic ectoderm (reviewed in Cohen, 1993). These primordia express Distal-less and are specified at the intersection between perpendicular

stripes of Wingless and Dpp, due to the cooperation between these two genes (Cohen, 1990; Cohen et al., 1993). Interestingly, the combined action of Wingless and Dpp also appears to direct Distal-less expression and specify organizer cell fate in the leg imaginal disc during larval development (see below).

1.5.2: Functions of Wingless during larval development

Wingless is expressed, and has a function, in all imaginal discs. In the leg disc, Wingless is expressed in a ventral-anterior wedge in cells just anterior to the A/P compartment boundary, under the control of Hedgehog (Basler and Struhl, 1994). Wingless is required in the leg disc both for P/D and D/V patterning. *wingless* mutations that reduce *wingless* function in the leg disc lead to the loss of ventral cell fates, and their replacement by dorsal ones (Baker, 1988; Couso et al., 1993). Also, ectopic expression of Wingless on the dorsal side of the disc is sufficient to induce ventral fates (Struhl and Basler, 1993; Diaz-Benjumea and Cohen, 1994; Wilder & Perrimon, 1995; Brook and Cohen, 1996; Jiang and Struhl, 1996). These results suggest that Wingless acts to specify ventral cell fates in the leg. It has recently been shown that Wingless also represses the Hedgehog-dependent expression of Dpp in the ventral half of the disc (Brook and Cohen, 1996; Jiang and Struhl, 1996), which may explain why ventral cell fates are replaced by dorsal ones in the absence of *wingless* function. Ectopic expression of Wingless on the dorsal side of the disc can also lead to a duplication of the P/D axis and hence a bifurcation of the leg, and may be due to the specification of a P/D organizing center in the cells that receive both the Wingless and the Dpp signal (Campbell et al., 1993; Struhl and Basler, 1993; Campbell and Tomlinson, 1995; Diaz-Benjumea et al., 1994). The expression and function of Wingless in the

antennal disc appears to be very similar to the leg disc (Diaz-Benjumea et al., 1994).

Wingless has several functions in the wing imaginal disc, which are temporally and spatially distinct, and which correlate with a complicated and dynamic expression pattern. Wingless expression in the wing disc is first detected in second instar in a ventral-anterior wedge similar to that found in the leg disc (Couso et al., 1993; Ng et al., 1996). Removal of wingless function at this point results in the loss of distal structures (the wing blade) and their replacement by a duplication of more proximal ones (the notum). This function of *wingless* is uncovered by the *wingless*^{1} regulatory mutation (Morata and Lawrence, 1977; Baker, 1987; van den Heuvel et al., 1993). Ng et al. (1996) have shown that ectopic expression of Wingless in the notum is able to induce a secondary wing, suggesting that Wingless is not only necessary, but also sufficient to specify the wing blade (which corresponds to the wing pouch in the wing disc). However, as already mentioned, at the time that Wingless induces wing pouch fate, it is expressed as a ventral-anterior wedge, and only cells at the apex of this wedge assume wing pouch identity, as assayed by the expression of *nubbin*, a gene expressed throughout the wing pouch (Ng et al., 1996). This suggests that Wingless must cooperate with another factor to induce nubbin expression and wing blade identity.

After its initial expression as an anterior-ventral wedge, Wingless is transiently expressed throughout the newly formed wing pouch, followed by an upregulation of Wingless expression at the D/V boundary and in a ring surrounding the wing pouch, and a concomitant downregulation in the rest of the wing pouch, in late second/early third instar (Phillips and Whittle, 1993; Couso et al., 1993; Ng et al., 1996). The addition of a second ring of Wingless surrounding the first, as well as a broad stripe running posteriorly to anteriorly across the notum in early/mid third instar completes the mature expression

pattern of Wingless in the wing disc (Couso et al., 1993; Phillips and Whittle, 1993; see also Fig. 3.2.2).

The function of *wingless* in several of these domains has been examined. At the D/V boundary, *wingless* activity is required for the expression of *acheate-scute* genes and for formation of wing margin bristles (Phillips and Whittle, 1993; Couso et al., 1994). Also, ectopic activation of the *wingless* pathway in the wing blade activates *acheate-scute* expression and leads to the formation of ectopic wing margin bristles (Simpson et al., 1988; Blair, 1992; Couso et al., 1994). While expression of low levels of Wingless has no effect on wing development (Struhl and Basler, 1993), strong ectopic expression of Wingless from a localized source not only leads to local respecification of cells to wing margin identity, but also causes surrounding cells to overproliferate (Diaz-Benjumea and Cohen, 1995). Taken together, these resuts suggest that Wingless may mediate both short-range and long-range effects of the organizing center at the D/V boundary. Data presented in chapters 5 and 6 of this thesis further address this possibility (see also Zecca et al., 1996; Neumann and Cohen, 1997).

Wingless activity is also required in the notum for the specification of several macrobristles which arise close to the stripe of Wingless expression in the notum (Phillips and Whittle, 1993). In the absence of *wingless* activity, the *acheate-scute* expressing proneural clusters of these bristles do not arise, suggesting that Wingless affects the pre-pattern that determines the location of specific bristles. It has also been shown that ectopic expression of Wingless in the notum can cause an increase in the density of bristles (Axelrod et al., 1996). However, this is probably a mechanistically different effect, as Axelrod and coworkers have proposed that it is due to inhibition of Notch signalling by Wingless. The increase in bristle density would thus be due to a reduction of lateral inhibition within existing proneural clusters, and not to the generation of new proneural clusters.

Chapter 4 of this thesis examines the function of *wingless* in one of the rings surrounding the wing pouch, and suggests that it may function as a localized mitogen in this part of the disc (see also Neumann & Cohen, 1996a).

In the eye imaginal disc, Wingless is expressed at the dorsal and lateral margins. Ma and Moses (1995) and Treisman & Rubin 1(995) have presented data suggesting that *wingless* activity is required in these regions of the eye disc to prevent ectopic initiation of a morphogenetic furrow. The morphogenetic furrow is normally initiated by Dpp at the posterior margin, and while loss of *wingless* activity leads to the ectopic initiation of morphogenetic furrows at the lateral margins, ectopic expression of Wingless suppresses the normal inititiation at the posterior margin. Wingless achieves these effects without affecting the expression of Dpp (which is expressed along the whole margin), suggesting that it may antagonize the effect of Dpp signalling.

It is interesting to note that Dpp and Wingless function antagonistically in some contexts, but synergistically in others. For example, Wingless and Dpp are expressed in adjacent domains in the visceral mesoderm of the embryonic midgut, and are both required for the activation of *labial* in nearby endodermal cells (Immerglueck et al., 1990; Panganiban et al., 1990). This is similar to the cooperation between Wingless and Dpp in the embryonic ectoderm to establish the thoracic imaginal disc primordia (Cohen, 1993) and the subsequent cooperation between Wingless and Dpp in the leg disc to establish a P/D organizing center at the center of the leg disc (Campbell et al., 1995; Diaz-Benjumea et al., 1994). However, at the same time, Wingless and Dpp repress each others expression in the leg disc, and there is also evidence suggesting that Dpp inhibits the ability of Wingless to activate *H15*, a Wingless target in the ventral leg (Brook and Cohen, 1996). Thus it appears that the interaction between Wingless and Dpp signalling can occur at several different levels, and can be both positive or negative, depending on the developmental context.

Wingless is also required for the development of the genitalia and the abdomen (Baker, 1988). Wingless is expressed in some of the precursor cells of the abdomen, the histoblasts, and is both necessary and sufficient to promote the formation of tergites and sternites, and also promotes the development of bristles in these structures (Shirras and Couso, 1996).

In conclusion, it is clear that wingless has many different functions both during embryonic and larval development, and that it affects both cell type specification (*eg.* at the wing margin) as well as proliferation (*eg.* in the Malphigian tubules). In some cases, Wingless functions as a permissive signal (*eg.* in bristle specification in the notum), while in others it functions as an instructive signal (*eg.* in bristle specification in the wing blade). Wingless can function both as a long-range morphogen (*eg.* in the wing pouch; see chapter 6 of this thesis and Zecca et al., 1996), or as a short-range signal (*eg.* in the induction of Distal-less expression together with Dpp in the embryonic ectoderm). It appears that in order to understand the diversity of responses to the Wingless signal in different contexts, it is necessary to understand how the competence of cells to respond to signals like Wingless is regulated.

1.6: The Wingless signalling pathway

Several components of the Wingless signalling pathway have been identified in Drosophila, including a candidate receptor. All genetically identified components of this pathway have been shown to be involved in all aspects of Wingless signalling examined so far. The components of the Wingless signal transduction cascade appear to be very similar to those functioning in the transduction of signalling by the Wnt-1 class of Wnts in vertebrates, indicating that this signalling pathway has been conserved during evolution.

Dfrizzled2

Dfrizzled2 is a member of the *frizzled* family of membrane-spanning proteins, and encodes a protein with seven putative transmembrane domains (Bhanot et al., 1996). *Dfrizzled2* was cloned on the basis of sequence similarity to *frizzled*, which has a function in controlling tissue polarity in Drosophila (Krasnow et al., 1995). Since the gene *dishevelled* functions both in Wingless signal transduction and in Frizzled-mediated control of tissue polarity (Klingensmith et al., 1994; Theisen et al., 1994; Krasnow et al., 1995) it was proposed that a frizzled-type protein could be the Wingless receptor (Bhanot et al., 1996). These authors show that transfection of *Dfrizzled2* into S2 cells confers responsiveness to Wingless protein (as assayed by the elevation of Armadillo protein), and that Wingless is able to bind to S2 cells expressing Dfrizzled2, but not to untansfected S2 cells. These results implicate Dfrizzled2 as the Wingless receptor, but it will be necessary to complement the biochemical with genetic data to fully make the case.

dishevelled

dishevelled was originally identified as an adult viable tissue polarity mutant, but it was later shown that removal of both maternal and zygotic *dishevelled* causes a segment

polarity phenotype in the embryo that is very similar to that of *wingless* (Perrimon & Mahowald, 1987). Genetic epistasis has since shown that *dishevelled* acts downstream of Wingless, but upstream of the *shaggy/zeste-white3* kinase, which functions as a negative regulator of Wingless signalling (Siegfried et al., 1994; Noordermeer et al., 1994). Consistent with the proposal that *dishevelled* is required for the reception of the Wingless signal, *dishevelled* activity is required cell-autonomously in order to respond to the Wingless signal (Couso et al., 1994; Klingensmith et al., 1994).

The *dishevelled* gene encodes a novel cytoplasmic protein that localizes to the plasma membrane (Klingensmith et al., 1994; Theisen et al., 1994; Yanagawa et al., 1995). Dishevelled is a phosphoprotein that becomes hyperphosphorylated in response to the Wingless signal (Yanagawa et al., 1995). Overexpression of Dishevelled is sufficient to cause its hyperphosphorylation in the absence of the Wingless signal, and to activate the Wingless pathway in cultured cells, as assayed by the accumulation of Armadillo protein (Yanagawa et al., 1995). Overexpression of Dishevelled is also able to activate the Wingless pathway *in vivo* (Axelrod et al., 1996; Neumann and Cohen, 1996a; Park et al., 1996; see also Chapter 4 of this thesis). However, while Axelrod and coworkers (1996) present data suggesting that overexpression of Dishevelled can only activate the Wingless pathway in the wing disc in the presence of Wingless protein, suggesting that Dishevelled overexpression only sensitizes cells to the Wingless signal, Park and coworkers (1996) show that overexpression of Dishevelled is sufficient to activate the pathway in a *wingless* null mutant background.

Axelrod et al. (1996) also present data suggesting that there is a negative cross-talk between the Wingless and Notch pathways, mediated by the binding of Dishevelled to the intracellular domain of Notch. Consistent with this proposal, Rulifson et al. (1996) have proposed that Wingless refines its own expression domain at the D/V boundary of the wing by

repressing the Notch-dependent activation of wingless..

Dishevelled homologues have recently been identified in mouse (Sussman et al., 1994) and in Xenopus (Sokol et al., 1995). As in Drosophila, overexpression of XDishevelled in frog embryos mimics ectopic expression of Wnts (Sokol et al., 1995), suggesting that XDishevelled functions in Wnt signal transduction.

shaggy/zeste-white3

The *shaggy/zeste-white3* gene has been shown to encode a serine/threonine kinase homologous to the vertebrate GSK-3 kinase (Bourouis et al., 1990; Siegfried et al., 1990, 1992; Ruel et al., 1993). In the absence of *shaggy/Zeste-white3* function, embryos behave as if the Wingless pathway is constitutively activated, evident both in the broadening of Engrailed expression, and in the naked phenotype of the ventral cuticle (Siegfried et al., 1992). This effect is independent of *wingless* function, suggesting that *shaggy/zeste-white3* functions as a negative regulator of the Wingless signal transduction pathway. Consistent with this proposal, *shaggy/zeste-white3* mutant clones in the wing blade autonomously activate Acheate-Scute expression and differentiate as margin bristles (Simpson et al., 1988; Blair, 1992).

Epistasis analysis has placed *shaggy/zeste-white3* downstream of *dishevelled* and upstream of *armadillo* in the Wingless pathway, suggesting that these genes may act in a simple linear sequence to transduce the Wingless signal (Siegfried et al., 1994; Nordermeer et al., 1994).

As in the case of Dishevelled, the Xenopus homologue of Shaggy/Zeste-white3, XGSK-3, appears to function in Wnt signal transduction. The overexpression of a dominant negative form of XGSK-3 induces similar responses in the frog embryo as injection of Wnt RNA (He et al., 1995; Pierce & Kimelman, 1995). Note also that it is the inactivation of XGSK-3 which triggers the Wnt pathway, suggesting that XGSK-3, like

Shaggy/Zeste-white3, is a negative regulator of Wnt signal transduction.

armadillo

armadillo encodes the Drosophila homologue of β-catenin, and was originally identified as a segment polarity mutant with a phenotype similar to *wingless* (Peifer & Wieschaus, 1990). As already mentioned, Wingless triggers the postranslational stabilization of Armadillo (Riggleman et al., 1990; Peifer et al., 1994). Armadillo is normally found at the cell membrane, with a small fraction in the cytoplasm, and it is the cytoplasmic pool of Armadillo which becomes stabilized in response to Wingless (Peifer et al., 1994), implicating this fraction in Wingless signal transduction.

While Armadillo/β-catenin also has a function in regulating cell adhesion, it has recently been proposed that this function is seperable from its function in Wnt signal transduction (Sanson et al., 1996). Consistent with this proposal, Orsulic and Peifer (1996), have shown that Armadillo is a modular protein, and that several of the domains of Armadillo are necessary either for the function in cell adhesion or in Wingless signalling, but not for both.

Like the other components of the Wingless signal transduction cascade, β-catenin also appears to function in Wnt signal transduction in vertebrates. For example, depletion of b-catenin mRNA using an antisense approach not only inhibits axis duplication in response to injected Wnts, but also endogenous axis duplication (Heasman et al., 1994), providing the first evidence that the Wnt signal transduction cascade is not only sufficient, but also necessary for normal D/V axis establishment in the Xenopus embryo. Data have recently been presented suggesting that the Wnt pathway is used to establish the Nieuwkoop center in the Xenopus embryo by activating expression of the homeobox gene *Siamois* (Carnac et al., 1996; Wylie et al., 1996).

As in Drosophila, Xenopus β-catenin appears to function downstream of XGSK-3 (Wylie et al., 1996), and it has recently been shown that XGSK-3 directly phosphorylates the N-terminus of β-catenin, and thereby inhibits it (Yost et al., 1996). Deletion of the N-terminus thus leads to the generation of a form of β-catenin that is constitutively active in Wnt signalling, both in Xenopus (Yost et al., 1996) and in Drosophila (Zecca et al., 1996).

LEF-1

Two independent studies indicate that a member of the LEF-1/Tcf-3 class of HMG box transcription factors mediates transcriptional activation by vertebrate Wnts (Behrens et al., 1996; Molenaar et al., 1996). These studies show that LEF-1 binds b-catenin, resulting in the nuclear translocation of β-catenin. While LEF-1 on its own does not activate transcription, the LEF-1-β-catenin complex does, suggesting that β-catenin functions as a transcriptional co-activator. These results are consistent with the observation that inhibition of XGSK-3 function leads to nuclear accumulation of β-catenin (Yost et al., 1996).

While microinjection of LEF-1 induces axis duplication in Xenopus embryos, injection of a dominant negative form suppresses both endogenous axis formation, and axis formation triggered in response to β-catenin injection (Behrens et al., 1996; Molenaar et al., 1996). Taken together, these results suggest that LEF-1 functions together with β-catenin to transduce Wnt signalling to the nucleus.

While these data only implicate LEF-1 in vertebrate Wnt signalling so far, the high degree of consevation of this signalling pathway between vertebrates and Drosophila suggests that a similar factor may play a similar role in Wingless signal transduction.

porcupine

porcupine was identified as a segment polarity gene with a *wingless* phenotype (Perrimon et al., 1989) and has been suggested to be required for the secretion of Wingless, as the Wingless protein appears to be confined to the expressing cells in *porcupine* mutants (van den Heuvel et al., 1993). Recently, the porcupine gene has been cloned, and shown to encode a protein with at least eight putative transmembrane domains that appears to localize to the endoplasmic reticulum (Kadowaki et al., 1996). In vitro, Porcupine not only affects secretion, but also glycosylation of Wingless protein. Consistent with the proposal that Porcupine is required for the production of the Wingless signal, *porcupine* mutant clones behave non-autonomously, just like *wingless* mutant clones (Kadowaki et al., 1996). These authors suggest that Porcupine may form a pore in the ER membrane, and thereby assist the post-translational translocation of Wingless into the lumen. An interesting aspect of this is that Porcupine is completely specific for Wingless, and does not affect any other secreted signals (Kadowaki et al., 1996).

In conclusion, a lot of progress has recently been made in unravelling the Wingless/Wnt-1 signalling pathway. All data obtained to date indicate that the same components are required to transduce the Wingless signal in all developmental contexts (for example, see Peifer et al., 1991; Blair, 1992, 1994; Couso et al., 1994; Diaz-Benjumea and Cohen, 1994; Klingensmith et al., 1994; Klingensmith & Nusse, 1994; Siegfried & Perrimon, 1994). It is also interesting to note the degree of conservation between the Wingless pathway in Drosophila and the Wnt-1 pathway in vertebrates, which explains why Wingless and Wnt-1 have practically identical effects on vertebrate cells (for example, see Chakrabarti et al., 1992; Ramakrishna and Brown, 1993).

CHAPTER 2:
MATERIALS AND METHODS

CHAPTER 2: MATERIALS AND METHODS

2.1: Recombinant DNA techniques

2.1.1: Restriction enzyme digestions

Typically, 0.5 - 5 μg of plasmid DNA was digested with 1 - 10 U of enzyme under the conditions recommended by the supplier. If a restriction digestion employing two enzymes was necessary, and the corresponding enzyme buffers were incompatible, the digestions were carried out sequentially. After the first digestion, the DNA was phenol extracted and precipitated as described in section 2.1.7 before proceeding with the second enzymatic treatment. When digestion of the vector DNA was carried out, the 5' phosphate groups were removed by adding 1 μl (10 U) of Calf Intestinal Phosphatase (CIP) to the reaction. CIP is active in all restriction enzyme buffers and can be heat-inactivated at 68°C for 10 min.

2.1.2: Ligation

Ligations were carried out in a volume of 10 μl using standard ligation buffer [50 mM Tris.HCl (pH 7.6), 10 mM $MgCl_2$, 4% PEG 8000 (w/v), 1 mM ATP, 1 mM DTT]. Typically 30 ng of vector DNA was ligated at 1:1 molar to insert with 5 U T4 DNA ligase. The reaction was carried out for either 3 hr at room temperature or for 30 min at 37 °C.

2.1.3: Gel purification of DNA fragments from agarose gels

The digested DNA fragments were run out on an agarose gel and the correct band was located within the agarose gel under UV light. The gel slice containing this fragment was cut out using a clean razor blade. The DNA was extracted from the gel slice using Geneclean II kit (BIO 101, USA) according to

manufacturer's instruction. The gel slice was weighed and transferred to an eppendorf tube. Three volumes of NaI solution (6 M NaI, saturating concentration of sodium sulphite) and 5 µl of glass milk were added to the tube. The tube was incubated at 55°C for 10 min and spun in a microcentrifuge for 10 sec. The glass milk pellet was re-suspended in 0.7 ml NEW wash solution (50% ethanol, 50 mM NaCl). The spinning and re-suspension were repeated two times. The pellet was air dried for 2 min and the DNA was eluted in 20 µl 1 x TE for 10 min.

2.1.4: Mini preparation of plasmid DNA

The alkaline lysis method was employed for mini preparation of plasmid DNA (Ausubel et al., 1994). A single bacterial colony was picked from a plate and grown in 2 ml of L-broth supplemented with the appropriate antibiotic overnight at 37°C with agitation. 1.5 ml of this bacterial suspension was transferred to an eppendorf tube and the tube was spun in a microcentrifuge for 30 sec. The pellet was then re-suspended in 100 µl alkaline lysis solution I [50 mM Glucose, 25 mM Tris.HCl (pH 8.0), 10 mM EDTA]. 200 µl of alkaline lysis solution II (0.2 N NaOH, 1% SDS) was added, the contents were mixed by inversion and the tube was placed on ice for 5 min. 150 µl of alkaline lysis solution III [5 M potassium acetate (pH 4.8)] was added, the tube was mixed by inversion and left on ice for 5 min. The supernatant was transferred to a fresh eppendorf tube and was then extracted with phenol/chloroform/isoamyl alcohol. The isopropanol-precipitated DNA was re-suspended in 100 ml of 1 x TE. Typically, 8 µl (about 1 µg) of DNA was used for restriction enzyme digestion.

2.1.5: Maxi preparation of plasmid DNA

Plasmid DNA was purified on a large scale using a Qiagen (Germany) maxiprep kit according to manufacturer's instructions. An overnight bacterial culture (about 0.5 ml) was used to inoculate 100 ml of L-broth supplemented with the appropriate antibiotic. The culture was allowed to grow overnight and the bacteria were harvested by centrifugation using a Beckman GS3 rotor (10 min at 7000 rpm and 4°C). The bacterial pellet was re-suspended in 10 ml of P1 [50 mM Tris.HCl (pH 8.0), 10 mM EDTA, 100 mg/ml RNase A] and transferred to a tube. 10 ml of P2 (0.2 NaOH, 1% SDS) was added. The contents were mixed by inversion and incubated on ice for 5 min. 10 ml of ice cold P3 [3 M potassium acetate (pH 5.5)] was added and the contents mixed again by inversion and icubated on ice for 5 min. The chromosomal DNA and bacterial debris were removed by centrifugation using a Sorvall SS-34 rotor (30 min, 10000 rpm, 4°C). The supernatant containing the plasmid DNA was loaded onto an equilibrated QIAGEN-tip 500. The contents of the tip were allowed to drain by gravity flow. The column was washed with 2 x 30 ml of QC buffer [1 M NaCl, 50 mM MOPS (pH7), 15% ethanol] and the plasmid DNA was eluted into a 30 ml corex tube with 15 ml of QF buffer [1.25 mM NaCl, 50 mM Tris.HCl (pH 8.5), 15% ethanol]. The plasmid DNA was precipitated and re-suspended in 0.5 ml 1 x TE. The quality and quantity of the DNA were determined spectroscopically by reading the optical densities at 260 nm and 280 nm.

2.1.6: Maxi-preparation of lambda phage DNA

Lambda phage DNA was purified on a large scale using a Qiagen (Germany) lambda DNA maxiprep kit according to manufacturer's instructions. 50 µl of phage solution was added to 0.5 ml of phage-competent HB101 bacteria. After incubation at room temperature for 15 min, this mixture was used to inoculate 250 ml of L-broth supplemented with 0.2% maltose

and 10 mM $MgSO_4$. After growing overnight at 37°C with agitation, 0.5 ml of chloroform were added and the culture was incubated for a further 30 min at 37°C. The bacterial debris was removed by centrifugation using a Beckman GS3 rotor (20 min at 7000 rpm and 4°C). 0.25 ml of buffer LI [20 mg/ml RNase A, 6 mg/ml DNase I, 0.2 mg/ml BSA, 10 mM EDTA, 100 mM Tris.HCl (pH7.5), 300 mM NaCl] were added to the supernatant. After incubating the tube at 37°C for 30 min, 50 ml of ice cold buffer L2 (30% PEG 6000, 3 M NaCl) were added. The tube was incubated on ice for 60 min. The phage-PEG pellet obtained by centrifugation at 10000 g for 10 min was re-suspended in 9 ml of buffer L3 [100 mM Tris.HCl (pH 7.5), 100 mM NaCl, 25 mM EDTA]. 9 ml of buffer L4 (4% SDS) was added and the contents were mixed by inversion. The tube was incubated at 70°C for 20 min and cooled on ice. 9 ml of buffer L5 [2.55 M potassium acetate (pH 4.8)] was added. The precipitated phage protein coat was removed by centrifugation at 15000 g at 4°C for 10 min. The supernatant containing the phage DNA was loaded onto an equilibrated QIAGEN-tip 500. The contents of the tip were allowed to drain by gravity flow. The column was washed with 30 ml of QC buffer. The phage DNA was eluted into a 30 ml corex tube with 15 ml of QF buffer, ethanol-precipitated, and re-suspended in 1 ml of 1 x TE. The quality and quantity of the DNA were determined spectroscopically by reading the optical density at 260 and 280 nm

2.1.7: Phenol extraction and precipitation of DNA

This procedure was carried out routinely to remove protein from DNA solution. The phenol/chloroform/isoamyl alcohol (25/24/1; v/v/v) solution was prepared according to manufacturer's instruction. 100 ml of phenol were mixed with 10 ml of 0.1 M Tris.base (pH 7) and left at room temperature for 2 hours. The Tris.base was then removed and 100 ml of

chloroform/isoamyl alcohol (24/1; v/v) were added. The resulting solution was stored at 4°C until use.

To de-proteinize a DNA solution, the following procedure was used. DNA solution was mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1; v/v/v) solution and spun 2 min in a microcentrifuge. The aqueous (upper) phase was transferred to a fresh eppendorf.

To precipitate the DNA, 2 mg of glycogen and 1/10th volume of 3 M sodium acetate (pH 5.2) were added to the DNA solution before the addition of 1 volume of isopropanol. After mixing, the solution was spun for 10 min. The supernatant was discarded and the precipitated DNA washed with 70% ethanol. The DNA pellet was air dried and resuspended in a suitable volume of 1 x TE.

2.1.8: Sequencing of DNA

All DNA sequence data were obtained from the EMBL sequencing service. DNA fragments subcloned into pBluescript were sequenced using the T7 and T3 primers.

2.1.9: Extraction of genomic DNA

Isolation of genomic DNA were carried out following standard procedures (Ausubel et al., 1994).

Typically, 50 - 100 flies were ground up in 3 ml HM [100 mM Tris.HCl (pH 8.0), 80 mM EDTA, 160 mM sucrose] in a homogenizer. The ground slurry was transferred to a fresh falcon tube and 750 µl 10% SDS and 500 µg proteinase K were added. The tube was incubated for 2 hr at 65 °C. The DNA was then phenol extracted, ethanol precipitated and resuspended in TE.

2.1.10: Plasmid rescue of P-elements

5 µg of genomic DNA from a strain carrying a P-element of the PZ class were digested with Xba I, or with Xba I in combination with Avr II, Spe I, or Nde I for several hours. This DNA was then phenol-chloroform extracted, ethanol precipitated and resuspended in 100 µl of 1 x TE. 10 µl of this DNA were ligated in a volume of 100 µl overnight at 18°C. Xba I cuts in the middle of the PZ construct and sequences to the right of this Xba I site contain a bacterial origin of replication and a Kanamycin resistance gene. Xba I (or one of the other three enzymes, which produce identical overhangs to Xba I) also cuts somewhere in the flanking genomic DNA, and circularization of this fragment due to the ligation of the two Xba I sites produces a plasmid that can be propagated in a bacterial host. The ligated mixture was once more phenol-chloroform extracted, ethanol precipitated and resuspended in 10 µl of water. All of this DNA was then used to transform bacteria and transformants were selected by plating out on an kanamycin-containing medium.

2.1.11: Southern blotting and hybridization

Standard procedures were employed for Southern blotting of agarose gels and for hybridization of the blots using radioactive DNA probes (Ausubel et al., 1994). The gel containing the size-fractionated DNA was soaked in 500 ml 0.25 N HCl for 15 min and then in 500 ml of 0.4 N NaOH for 30 min. The gel was trimmed at one corner for orientation purposes. All the required components for blotting were soaked in 0.4 N NaOH. For blotting DNA with a high degree of complexity (*eg.* genomic DNA), a standard setup was used. The gel was placed upside-down on two layers of Whatman 3 MM papers which in turn were placed on a clean sponge partly submerged in a tray of 0.4 N NaOH solution. A piece of GeneScreen Plus nylon membrane (DuPont, USA) trimmed to the size of the gel was placed on top. Care was taken to ensure

that no air bubble was trapped between any of the layers. A small weight was placed on a pile of of blotting papers which were placed on top of the nylon membrane. The whole setup was left undisturbed overnight. Transfer of DNA from the gel onto the membrane was achieved by capillary action. After the transfer, the blot was rinsed in 50 ml of 2 x SSC and pre-hybridized in 100 ml of hybridization solution (50% formamide, 5 x Denhardt's, 5 x SSC, 1% SDS) at 42°C for > 1 hour. Hybridization was carried out overnight in 5 ml of hybridization solution containing a heat-denatured radioactive DNA probe (> 10^7 cpm). The next day, the blot was washed with 100 ml pre-warmed 1 x SSC, 0.1% SDS at 65°C for 20 min. The wash was repeated two times. The membrane was wrapped in plastic wrap and exposed to X-ray film.

2.1.12: Plaque lifting and screening

Standard techniques were used to identify a desired recombinant phage clone on a plate (Ausubel et al., 1994). The plates containing plaques were chilled at 4°C for > 2 hours. For a 95 mm plate, a circular 90 mm nylon membrane was laid on the top agarose for 3 min. Using a gauge 16 needle, four holes were pricked through the membrane and the agar for orientation purposes. For a 234 mm square plate, two pieces of 116 x 225 mm nylon membranes were used. After the lift, the membrane was submerged in each of the following buffers for 30 sec and then air dried: buffer A (1.5 M NaCl, 0.5 M NaOH), buffer B [1.5M NaCl, 0.5 M Tris.HCL (pH 7.5)], and 2 x SSC. Pre-hybridization, hybridization, washing and exposure of the membrane was carried out as described for Southern blotting. The positive signals on the X-ray film were used to locate the corresponding plaques on the plate. An agar plug corresponding to each of the positive plaques was removed using a cut-end blue pipette tip and transferred to an eppendorf containing 1 ml of SM and 20 µl chloroform. Elution of the phages from the agarose plug was allowed to occur at

room temperature for 2 hours. The phage solution was either plated out for a re-screen, or used to inoculate a culture for maxi-preparation of phage DNA.

2.2: Probe labelling procedures

2.2.1: Random prime radioactive labelling of double stranded DNA

Double stranded DNA used for Southern blot hybridisation was radioactivelly labelled with ^{32}P-α-dCTP using the multiprime DNA labelling system (Amersham, USA) according to manufacturer's instructions. 20 ng (11.5 µl) of DNA were mixed with 2.5 µl random hexamer and heat-denatured at 95°C for 5 min. The tube was allowed to cool to room temperature, 5 µl of deoxyribonucleotide mix, 5 µl of ^{32}P-α-dCTP, and 1 µl of Klenow were added. The labelling was carried out at 37°C for 1 hour. Unincorporated nucleotides were removed by gel filtration using a sepharose G-50 column (Boehringer Mannheim, Germany) according to manufacturer's instructions. The sepharose beads in the column were re-suspended in the storage buffer and allowed to pack under the ingluence of gravity. The storage buffer was drained from the column by gravity flow. The column was spun at 2000 g for 2 min. The probe was diluted to 100 µl with ddH_2O and loaded onto the pre-spun column. The column was spun again at 2000 g for 4 min. The flow-through contained the clean probe. The efficiency of labelling was typically 1 x 10^9 cpm/µg DNA.

2.2.2: Digoxigenin labelling of riboprobes

Digoxigenin labelled riboprobe was prepared using DIG RNA labelling kit (Boehringer Mannheim, Germany) according to manufacturer's instruction. In vitro transcription reactions were carried out in a volume of 20 µl containing 1 µg of

linearized plasmid, 2 µl of 10 x transcription buffer [0.4 M Tris.HCl (pH 7.5), 60 mM $MgCl_2$, 0.1 M NaCl, 20 mM spermidine], 2 µl of 10 x ribonucleotide mix (containing DIG-UTP, ATP, CTP, GTP), 1 µl of "RNA guard", and 2 µl of T7 or T3 RNA polymerase. The tube was incubated at 37°C for 2 hours. After labelling, 1 µl of the mixture was run on an agarose gel to assess the success of the reaction, and 25 µl of 2 x carbonate buffer (120 mM Na_2CO_3, 80 mM $NaHCO_3$) was added to the remaining probe and the tube was incubated at 65°C for 20 min. The probe was precipitated with glycogen and resuspended in 150 µl of in situ hybridisation buffer (E-hyb).

2.2.3: Digoxigenin labelling of DNA probes

Typically, 100 - 400 ng of DNA were heat denatured at 95°C for 5 min and annealed to 30 µg of random hexamer in a total volume of 16 µl. 2 µl of 10 x buffer [1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM digoxigenin 11-dUTP (Boehringer Mannheim, Germany)] and 5 U of Klenow were added. The tube was incubated overnight at 37°C and then diluted to 100 µl with E-hyb. The tube was stored at -20°C.

2.3: Bacterial techniques

2.3.1: Bacterial strains

The *E. coli* HB101 [genotype: F$^-$ D(*gpt-proA*)*62 leu supE44 ara14 galK2 lacY1* D(*mcrC-mrr*) *rpsL20* (Str^r) *xyl-5 mtl-1 recA13*] and XL-1 blue [genotype: F'::Tn*10 proA*$^+$*B*$^+$ *lacI*^{q} D(*lacZ*)*M15/recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* (rK^-mK^+) *supE44 relA1 lac*] strains were used for the propagation of double stranded plasmids.

2.3.2: Maintenance and Media

E. coli cells were grown at 37 °C in L-broth or on L-broth agar plates as described. Ampicillin or kanamycin (100 µg/ml) was added when necessary.

2.3.3: Preparation of competent cells and transformation of *E. coli*

Calcium chloride mediated transformation of bacteria was used. Briefly, an overnight culture of bacteria was diluted hundred fold into 100 ml L-broth media and 10 mM $MgCl_2$. The bacterial culture was grown to a density corresponding to 0.3 units at 600 nm. All subsequent steps were carried out at 4 °C. The culture was transferred to two pre-chilled 50 ml falcon tubes and spun at 2000 rpm for 10 min. The pellets were resuspended in 5 ml of cold 100 mM $CaCl_2$, stored on ice for 1 hr, and then spun 10 min at 2000 rpm. The pellet was resuspended in 5 ml 100 mM $CaCl_2$ and 20% glycerol, aliquoted in 250 µl portions and frozen in a dry ice/ethanol bath and stored at - 80 °C. 100 µl of cells were used per ligation. Cells were thawed on ice and mixed with ligated DNA (about 70 ng). The tube was incubated on ice for 45 min, heat shocked at 42°C for 90 sec, and returned to 4°C for 1 min. 1 ml L-broth media was added to the cells and the bacteria allowed to grow with gentle shaking at 37°C for 30 min. The bacteria was spun down in an eppendorf centrifuge for 4 min at 7000 rpm, resuspended in 100 ml L-broth media and plated out on LB plates containing the appropriate antibiotics.

2.3.4: Preparation of plating bacteria and plating out lambda phages

The HB101 bacterial strain was used to propagate λDASH II phages. Standard procedures were used to prepare plating bacteria and to plate out phages (Ausubel et al., 1994). A single bacterial colony was used to inoculate 50 ml of L-broth

supplemented with 0.2% maltose and 10 mM $MgSO_4$. After growing overnight at 37°C with agitation, the bacterial suspension was transferred to a 50 ml facon tube and spun at 2500 rpm for 10 min using a Sorvall RC-3B rotor. The bacterial pellet was resuspended in 25 ml of 10 mM $MgSO_4$. The HB101 strain prepared in this way could be stored at 4°C for 7 days.

Typically, a total number of 1 x 10^6 - 5 x 10^6 phages were plated out for screening a genomic library. Two 234 mm x 234 mm LB plates were used. For each of these plates, 500 ml of plating bacteria were mixed with 1 µl - 200µl of phage-containing solution in a 50 ml Falcon tube and incubated for 15 min at room temperature. 30 ml of molten top agarose (48°C) containing 10 mM $MgSO_4$ were added. After mixing, the contents of the tube were poured onto a pre-warmed LB plate. The top agarose was allowed to set for 10 min at room temperature and the plate was incubated upside-down in a 37°C incubator for about 12 hours. On the next day, confluent phage plaques were found to cover the plate, and the phages were transferred onto a piece of nylon membrane for screening (see section 2.1.12). A very similar procedure, with the following modifications, was used for re-screening of phage clones. A series of dilutions (typically 1 x 10^{-3} to 1 x 10^{-6}) of a phage-containing solution was made. A 95 mm circular plate was used to plate out each of these dilutions. The phages were mixed with 200 ml plating bacteria in a Falcon 2059 tube, incubated for 15 min at room temperature, mixed with 3 ml of molten agarose, and then plated out. After overnight incubation at 37°C, the plate found to contain 30 - 300 phage plaques was used for plaque lifting (section 2.1.12).

2.4: Electrophoresis

2.4.1: Agarose gel electrophoresis (AGE)

The required weight of agarose was dissolved in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) by boiling the agarose for several minutes. The gel mould was prepared and before pouring the agarose solution, ethidium bromide was added to a final concentration of 0.05 mg/ml. Prior to loading, the samples were mixed with 1/6th volume of gel loading buffer [20% (w/v) Ficoll, 1 mM EDTA (pH 8.0), 0.05% (w/v) bromophenol blue, 0.1% (w/v) SDS]. The gels were submerged and electrophoresed in 1 x TAE buffer containing 0.05 mg/ml ethidium bromide at 15 V/cm. The DNA fragments were visualised by UV irradiation.

2.5: Analysis of polytene chromosomes

2.5.1: *In situ* hybridization to polytene chromosomes

The salivary glands from third-instar larvae were dissected in PBS and fixed in a drop of 45% acetic acid on a clean, siliconized 18 mm x 18 mm coverslip for 5 - 10 min. The coverslip was placed on a glass slide and the polytene chromosomes were spread by stroking the surface of the coverslip with a stiff needle. Excess acetic acid was blotted off and the slide was examined under a phase contrast microscope. Stroking was repeated if the spreading of the chromosomes was insufficient. The slides were frozen on dry ice and stored at -80°C until use. Before staining, the coverslip was quickly flipped off using a razor blade and the slide was immediately immersed in freshly prepared ethanol/acetic acid (3/1; v/v) for 10 min, dehydrated in 96% ethanol for 10 min, and air dried. The chromosomes were heat-denatured in 2 x SSC at 58°C for 30 min, and allowed to cool down in 2 x SSC for

2 min. The chromosomes were dehydrated by immersing the slide in 70% ethanol for 2 x 5 min, 95% ethanol for 5 min, and air dried. The chromosomes were denatured by immersing the slide in freshly made 0.7 N NaOH (pH 12.5) for 3 min, followed by neutralization in 2 x SSC for 3 x 5 min. The chromosomes were once more dehydrated and air dried as before. 3 ml of heat-denatured DIG DNA probe was mixed with 27 µl of E-hyb and added to the slide. The slide was covered with a 22 mm x 22 mm coverslip, and incubated overnight at 53°C in a moist chamber. The next day, coverslip was removed and the slide was washed with PBT for 3 x 2 min. Alkaline phosphatase-conjugated mouse anti-DIG antibody (Boehringer Mannheim) was diluted 200-fold with PBT and 200 µl of this mixture was added to the slide. The slide was covered with a 22 mm x 40 mm coverslip and incubated at room temperature for 1 hour. The slide was washed with PBT for 3 x 3 min and with AP buffer for 3 x 3 min. 4.5 µl NBT, 3.5 µl BCIP, and 1 ml of AP buffer were mixed. 200 µl of this mixture were added onto the slide. The colour was allowed to develop in a moist chamber for several hours. When the desired intensity of staining was reached, the slide was washed with dd H_2O for 3 x 3 min, counter-stained with Giemsa [5% Giemsa (Sigma), 0.1 M phosphate buffer (pH 6.8)] for 100 sec, de-stained under running dd H_2O for 2 min, air dried, and embedded in 30 µl Permount (Sigma).

2.6: Histochemical methods

2.6.1: *In situ* hybridization to imaginal discs

Whole mount in situ hybridization to imaginal discs using digoxigenin labelled RNA probes was carried out as described by (Tautz & Pfeifle 1989). Larvae were dissected in PBS, fixed for 20 min with 4% formaldehyde (Polyscience, USA) in PBS, washed with PBT2 (PBS, 0.2% Tween 20) three times, and

digested with 10 mg/ml Proteinase K in PBT2 for 1 - 5 min. The digestion was stopped by washing the discs with 2 mg/ml glycine in PBT2 for 2 min, followed by two washes in PBT2. The discs were then post-fixed with 4% formaldehyde + 0.2% glutaraldehyde in PBT2, and washed three times with PBT2. The discs were then washed in a 1:1 mix of PBT2 and E-hyb, and pre-hybridized with E-hyb at 55°C for 1 hour. 2 ml of DIG-labelled probe in 50 ml of E-hyb were added to the discs and hybridization was allowed to occur overnight at 55°C. The next day, the discs were washed first with E-hyb, then with a 1:1 mix of E-hyb and PBT2, and finally four times with PBT2. Alkaline-phosphatase conjugated mouse anti-DIG antibody (Boehringer Mannheim, Germany) was added to the discs at 1:2000 dilution in PBT2. After incubation for 1 hour, the discs were washed four times with PBT2 and two times with AP buffer [0.1 M Tris.HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$, 0.1% Tween 20]. The colour was developed in a small weighing dish containing 4.5 µl of NBT (70% DMF, 100 µg/ml NBT), 3.5 µl of BCIP (100% DMF, 50 µg/ml BCIP), and 1 ml of AP buffer in the dark. When the desired staining intensity was reached, the reaction was stopped by washing several times with PBT2. The discs were equilibrated in glycerol mount (80% glycerol, 1 x PBS) and dissected.

To compare labelling of wild type and mutant discs as directly as possible, fixation, hybridization and labelling reactions were carried out in parallel, or, if the mutant phenotype was clearly distinguishable from that of wild-type discs, in the same tube.

2.6.2: X-gal staining

Larvae were dissected in PBS and fixed for 5 min in 1% glutaraldehyde in PBS (Fluka, USA), washed three times in PBX2 (PBS, 0.2% Triton X-100) and incubated in X-gal staining solution [1 x PBS, 5 mM $K_3(Fe^{II}(CN)_6)$, 5 mM $K_4(Fe^{III}(CN)_6)$, 0.3% Triton X-100, 8% X-gal] at 37°C on a shaking platform.

When the desired staining intensity was achieved (typically 2 hours to overnight), the larvae were washed 3 times in PBX2 and equilibrated in glycerol mount (1X PBS, 80% glycerol).

To stain adult flies, newly (< 1 hour) eclosed individuals were used. The wings were removed in PBS, fixed with 1% glutaraldehyde in PBS for 5 min, washed three times with PBX100 (PBS, 10% Triton X-100), and incubated in X-gal staining solution overnight. The wings were washed three times with PBX2, equilibrated in glycerol mount, and mounted.

2.6.3: Acridine orange staining

Acridine orange staining was done as described by (Masucci et al., 1990). Larvae were dissected in PBS and kept in ice cold PBS for less than 30 min. The discs were incubated acridine orange solution (100 mM acridine orange, 1 x PBS) for 5 min in the dark, and washed two times with PBS for 3 min in the dark. The discs were then dissected from the larval heads, mounted in a drop of 1 x PBS on a microscope slide, covered with a coverslip spotted with Vaseline at each of the corners, and examined under a fluorescence microscope.

2.6.4: BrdU labelling of imaginal discs

BrdU labelling of discs was performed as described in Usui & Kimura (1992). Larvae were dissected in PBS and then placed in PBS containing 200 µg/ml BrdU. The discs were incubated in the BrdU solution for 30 min to 2 hours and then fixed in modified Carnoy's for 15 min. The discs were rehydrated in an ethanol series (70%, 50%, 25%, in PBS) and then washed several times in PBX2. The chromosomes were denatured for 30 min in 2N HCl (to allow access of the antibody). The discs werer washed several times in PBX2 and blocked for 1 hour with 10% NGS in PBX2. a-BrdU (Amersham) was added at a 1/20 dilution and incubated for 2 hours at room temperature, or overnight at 4°C. The discs were washed

several times in PBX2 with 4% NGS. Secondary goat α-mouse antibody was added and the remaining procedure was the same as for a normal antibody stain (see 2.6.5).

For double-labelling discs with BrdU and X-gal, the protocol was modified in the following manner (Usui and Kimura, 1992). After incubation in BrdU solution, the discs were fixed in 0.04% glutaraldehyde for 15 min and then washed several times in PBX2. The discs were incubated in X-gal staining solution until the desired intensity of staining was reached, and were then washed several times in PBX2. The discs were post-fixed in modified Carnoy's for 15 min, and the rest of the procedure was the same as for normal BrdU labelling.

2.6.5: Antibody staining to imaginal discs

Larvae were dissected in PBS, fixed with 4% formaldehyde (Polyscience, USA) in PBS for 20 min, and washed five times with 4% NGS in PBT3 (PBS, 0.3% Tween 20). Primary antibody was added to the discs at the desired concentration in the same solution, and the contents were incubated overnight at 4°C. The discs were washed five times with 4% NGS in PBT3. Secondary antibody was added to the discs at a 1:500 dilution, and the contents were incubated for 1 hour at room temperature. For histochemical staining, biotinylated secondary antibody and a Vectastain elite ABC kit (Vector laboratory, USA) were used. The discs were washed four times with PBT3, and incubated with premixed ABC solution (5 ml elite A, 5 ml elite B in 500 ml PBT3, pre-incubated for at least 15 min) for 30 min. After washing the discs four times with PBT3, the colour was developed in a small weighing dish containing 1 ml DAB staining solution (0.5 μg/ml DAB, 0.3% H_2O_2, PBT3). When the desired intensity of staining was reached, the reaction was stopped by washing the discs several times with PBT3. The discs were equilibrated in glycerol mount and dissected. For fluorescent staining, FITC- or Texas Red-conjugated secondary antibody was used. The discs

were washed four times with PBT3 in the dark, and mounted in nPG-glycerol (80% glycerol, 4% n-propylgalate, 1 x PBS).

2.6.6: Antibodies used in this study

A list of the primary and secondary antibodies used in this study is given below.

Antibodies	Source/Reference
Primary antibodies (final concentration)	
monoclonal mouse α-Wingless (1:10)	(Brook and Cohen, 1996)
polyclonal mouse α-Distal-less (1:500)	(Vachon et al., 1992)
polyclonal rabbit α-Engrailed (1:100)	P. O'Farrell
polyclonal rabbit α-Vestigial (1:100)	(Williams et al., 1991)
polyclonal rabbit α-Ci (1:10000)	(Johnson et al., 1995)
polyclonal rat α-Dishevelled (1:250)	(Yanagawa et al., 1995)
monoclonal mouse α-Myc (9E10) (1:1000)	K. Bohmann
polyclonal rabbit α-β-galactosidase (1:5000)	Cappel
alkaline phosphatase-conjugated mouse α-digoxigenin (1:2000)	Boehringer Mannheim
monoclonal mouse α-BrdU (1:20)	Amersham
Secondary antibodies (all used at 1:500)	
biotinylated goat α-mouse	Jackson laboratory
biotinylated goat α-rabbit	Jackson laboratory
texas red-conjugated goat α-mouse	Jackson laboratory
texas red-conjugated goat α-rabbit	Jackson laboratory
texas red-conjugated goat α-rat	Jackson laboratory
FITC-conjugated goat α-mouse	Jackson laboratory
FITC-conjugated goat α-rabbit	Jackson laboratory

2.7: Drosophila stocks

2.7.1: Culture conditions

Flies were cultured on standard Drosophila medium (1% agar, 9% corm flour, 0.4% propionic acid, 2.5% sucrose, 1.5% yeast extract) in 20 mm x 95 mm shell vials or in 40 mm x 100 mm plastic bottles at 25°C. Larvae were fed with plenty of live yeast under uncrowded conditions. Staging of larvae was performed both by total incubation time and by examination of the larval anterior spiracles (Ashburner, 1989).

2.7.2: Strains generated by transformation

All transgenic lines presented in this study were generated by Ann-Mari Voie. P[w^+] DNA constructs were introduced into the germ line of w^{1118} flies by standard P-element mediated transformation (Rubin & Spradling, 1983). Briefly, 3 µg of a P[w^+] DNA construct was mixed with 0.5 µg of helper DNA (coding for Δ2-3 transposase), ethanol precipitated, and re-suspended in 10 µl of injection buffer [5 mM KCl, 0.1 mM phosphate buffer (pH6.8)]. This mixture was injected into the posterior pole of pre-blastoderm embryos. The resulting flies were individually mated with w^{1118} flies. Transformants carrying the transgene were identified in the next generation (F1) based on the rescue of white eye colour by the w^+ construct. Typically, 60 - 200 embryos were injected to obtain at least one G0 fly bearing a < 30 kb transgene.

2.7.3: Strains generated by P-element mediated mutagenesis

P-elements are transposons that can be used to mutagenize genes in two ways: firstly, the insertion of a P-element into a gene can lead to its inactivation (*eg.* see wg^{r0727}, Fig. 3.2.7); secondly, the imprecise excision of a P-

element can cause the deletion of flanking DNA, and thereby generate a mutation (Engels et al., 1990).

The insertional mutagenesis approach was used to revert the *Sternopleural* (*Sp*) dominant phenotype (section 3.3.3). Tower et al. (1993) and Zhang and Spradling (1993) have presented data suggesting that P-elements preferentially transpose to nearby genomic locations ('local hops'). wg^{r0727} is a P-element of the PZ class inserted into the 5' region of the *wingless* gene (see Fig. 3.2.7). Since the mutation *Sp* maps very close to the *wg* gene, I decided to use wg^{r0727} (which is viable and fertile when crossed to *Sp*) to revert the *Sp* dominant phenotype by local hop. To do this, wg^{r0727} was crossed to *Sp* together with the *Δ2-3(99B)* insertion on the third chromosome (*Δ2-3(99B)* is an engineered form of P-element that constitutively produces transposase, but cannot transpose itself), in the hope that the P-element would jump from the wg^{r0727} chromosome onto the *Sp* chromosome, and that one such event might might lead to the inactivation of the gene causing the *Sp* dominant phenotype. The genotype in which the jump occurred was: wg^{r0727}, *b pr cn/Sp; Δ2-3 Sb/+*. These males were crossed to *l(2)/CyO* virgins. The wg^{r0727}, *b pr cn* chromosome can be identified when crossed to the *CyO* balancer because *CyO* also carries the *pr* and *cn* mutations (in addition to the dominant *Cy* mutation), which give an eye-colour phenotype. It was thus possible to screen for *Sp* chromosomes over *CyO* (*ie.*, the flies that had *Cy* wings and wild-type eye-colour) and to select for individuals that had lost the *Sp* dominant phenotype. Out of 2000 F1 progeny, one such phenotypic *Sp* revertant was recovered and termed Sp^{revP}. Subsequent analysis indicated that the Sp^{revP} P-element had inserted into the *wg* 3' UTR (section 3.3.3).

The excisional mutagenesis approach was used to generate *Df(2L)spd*^{i2} and *Df(2L)spd*^{hL2}. Again, the wg^{r0727} P-element was used as the starting point. The males in which excision took place were of the genotype: wg^{r0727}*/Sco; Δ2-3 Sb/+*. These were crossed to *l(2)/CyO* virgins. All stocks used

for this screen werer kept in a *rosy* mutant background. Since the PZ element carries a $rosy^+$ transgene, this allowed the detection of individuals in which mobilization of the P-element had led to complete or partial loss of the P-element, by screening for $rosy^-$ flies. Several such events were recovered, and these were further analyzed by crossing them to *spdfg*. A precise excision event would generate a chromosome that complements the *spdfg* mutation, while an imprecise excision might generate a mutation that fails to complement *spdfg* (wg^{r0727} only shows a very weak wing phenotype over *spdfg*). Two imprecise excicions that failed to complement *spdfg* were recovered, and termed $spdj^2$ and spd^{hL2}. Subsequent analysis showed that both of these mutations are due to deficiencies generated by imprecise excision (see section 3.2).

2.7.4: Genetic mosaic analysis

The generation of mosaic imaginal discs was done using the FLP/FRT technique as described by Xu & Rubin (1993). This approach makes use of the site-specific FLP recombinase to catalyze recombination of its target sequences (called FRT sites) located on homologous chromosome arms. For this experimental approach, a mutation is recombined onto a chromosome carrying an FRT site between the gene of interest and the centromere, and this is crossed to a chromosome carrying the same FRT and a cell marker (*eg. N-myc*) distally to the FRT, together with a separate chromosome bearing a hs-*flp* construct. Heat-shock induced expression of the FLP enzyme then causes mitotic recombination between the FRTs in a random population of cells (the efficiency can be controlled by the severity of the heat shock). This leads to the generation of patches of cells which lack expression of the marker gene and which are also homozygous mutant for the gene of interest. These clones can then be assayed for the expression of other genes for which probes are available.

The FLP/FRT chromosomes used in this study are listed below.

Genotype	Source/Reference
hsFLP chromosomes	
y w hs-*flp*	(Struhl and Basler, 1993)
y hs-*flp f*	(Diaz-Benjumea and Cohen, 1995)
FRT chromosomes	
$arm^{H8.6}$ FRT101	(Orsulic and Peifer, 1996)
y w dsh^{VA153} FRT18A	(Theisen et al., 1996)
y w dsh^{75} FRT101	(Rulifson et al., 1996)
y w dsh^{v26} FRT 101	(Axelrod et al., 1996)
wg^{CX4} FRT40A	(F. Diaz-Benjumea; Neumann and Cohen, 1996b)
$Su(H)^{SF8}$ FRT40A	(Schweisguth, 1995)
N-myc FRT40A	(Xu and Rubin, 1993)
arm-lacZ FRT40A	(Wiersdorff et al., 1996)
N-myc FRT101; hs-*flp* MkRS/*Tm6, Sb*	(Neumann and Cohen, 1997)
N-myc FRT18A; hs-*flp* MkRS/*Tm6, Sb*	(Xu and Rubin, 1993)

2.7.5: A gain of function assay using the flip-out system

The flip-out system was designed to generate randomly positioned clones of cells expressing a gene of interest (Struhl and Basler, 1993). This technique requires two fly strains: a strain carrying a hs-*flp* transgene and a strain carrying a flip-out transgene. The hs-*flp* line, and the principle of FLP-mediated recombination of FRT sites has been described in

section 2.7.4. When two FRTs are arranged as direct repeats, Flp-mediated recombination leads to excision of the intervening DNA and joining of the sequences on either side. The flip-out construct consists of a flip-out cassette separating a cDNA and a constitutive promoter. The flip-out cassette consists of a transcriptional terminator and a marker gene flanked by two FRTs oriented in the same direction. Therefore, the coding sequence of the cDNA in the flip-out construct will not be expressed unless the flip-out cassette containing the transcriptional terminator is excised by Flp-mediated recombination. In animals carrying both the hs-*flp* and the flip-out transgenes, a brief pulse of ubiquitous Flp expression driven by heat-shock induced activation of the hs-*flp* gene will lead to to excision of the flip-out cassette and to expression of the cDNA. The efficiency and hence the frequency of clones expressing the gene of interest can be controlled by varying the severity of the heat shock applied.

The only flip-out transgene used in this study is a *Ubx>f*$^{+}$*>wg* construct, in which a *forked*$^{+}$ cDNA is inserted in the flip-out cassette that separates the *Ubx* promoter from a *wingless* cDNA (Diaz-Benjumea and Cohen, 1995). The *forked*$^{+}$ cDNA partially rescues the bristle phenotype of *forked* mutants. If the flip-out event is generated in a *forked* mutant background, it is thus possible to identify the cells in which the *wingless* cDNA is activated, as these will fully express the *forked* mutant phenotype. The flip-out events described in this study (section 3.3.3) were induced by a 33°C, 25 min heat shock during second instar.

2.7.6: A gain of function assay using the GAL4:UAS system

The GAl4:UAS system was designed for persistent targeted expression of any cloned gene in a spatially restricted manner (Brand & Perrimon, 1993). This technique also requires two fly strains: a GAL4 driver strain and a UAS-cDNA responder strain. The driver strain carries transgene

expressing the yeast GAL4 transcriptional activator in a spatially defined pattern. This can be achieved by placing a specific ehancer element upstream of the GAL4 coding sequence before transformation. Alternatively, a GAl4 transgene equipped only with a basal promoter is allowed to integrate randomly into the genome, and insertions are identified that express GAL4 in distinct patterns under the control of nearby genomic enhancers (Brand and Perrimon, 1993). The responder strain carries a transgene expressing a gene of interest under the control of five GAL4 binding sites, termed an upstream activating sequence (UAS). Therefore, a fly strain in which a driver and a responder strain are brought together will mis-express the target gene in a spatially defined pattern. The level of expression of the target gene can be modulated somewhat by raising the animals at different temperatures. This is because the transactivation potential of the GAL4 protein appears to be higher at higher temperatures (Wilder and Perrimon, 1995). In animals carrying a UAS-reporter construct (*eg.* UAS-*lacZ*) in addition to the driver and responder constructs, the cells mis-expressing the gene of interest can be identified due to the presence of the reporter protein.

The fly strains used for GAL4:UAS experiments in this study are listed below:

Genotype	Chromosome	Source/Reference
Driver Strains		
MS1099-GAL4	X	(Capdevila & Guerrero, 1994)
dpp-GAL4	III	(Wilder and Perrimon, 1995)
vg-GAL4	II	(Simmonds et al., 1995)

Responder strains

UAS-*wingless*	III	(Lawrence et al., 1995)
UAS-*dishevelled*	II + IV	(Neumann and Cohen, 1996a; This thesis)
UAS-*Notch(intra)*	III	(M. Haenlin; Neumann and Cohen 1996b)
UAS-*vestigial*	II	(Kim et al., 1996)
UAS-*lacZ*	II	(Brand and Perrimon, 1993)

UAS-*dishevelled* was constructed by cloning a full-length *dishevelled* cDNA (Klingensmith et al., 1994) as an *Eco*RI fragment into the *Eco*RI site in the multiple cloning sequence in pUAST (Brand and Perrimon, 1993). This places the cDNA downstream of the UAS sequence (the orientation was checked using *Kpn*I), and this construct was used to transform flies.

2.7.7: An assay for enhancer activity in Drosophila

Enhancer activity of a fragment of genomic DNA in Drosophila can be tested by cloning it upstream of a reporter gene, and assaying the expression of this reporter gene after it has been transformed into flies (Thummel & Pirrotta, 1991). The 1.2 kb *spd* enhancer described in section 3.2.2 was tested by cloning it as an *Eco*RI fragment into the multiple cloning site placed upstream of the hs43 promoter in the hs43-AUG-bGal vector (CHAB, Thummel and Pirrota, 1991). Digestion with *Hind*III showed that it had been cloned in the sense orientation (not shown). This construct was used to transform flies, and transformants were tested for enhancer activity with X-Gal staining (section 3.2.2).

2.7.7: Preparation of adult cuticle for microscopic examination

Adult cuticle preparations were carried out as described by Lawrence et al. (1986). Adults were stored in SH solution (70% glycerol, 30% ethanol) and were then dissected, rinsed in dd H_2O, boiled in 10% KOH, rinsed in dd H_2O again, and dehydrated in ethanol. The dissected cuticle was mounted in a mixture of 60% lactic acid and 40% ethanol. The microscope slides were sealed with nail polish.

2.7.8: The Drosophila strains used in this study

Most of the fly strains used in this study ar described in Lindsley & Zimm (1992). Those that are not are described in the results section. All the strains used in this study are listed below (except for FRT strains and GAL4/UAS strains, which are listed in sections 2.7.4 and 2.7.6, respectively).

Genotype	Source/Reference
wingless (*wg*) alleles	
spade^{flag} (*spdfg*)	(Lindsley and Zimm, 1992; Neumann and Cohen, 1996a; This study)
Sternopleural (*Sp*)	(Lindsley and Zimm, 1992; Neumann & Cohen, 1996c; This study)
wg^{CX4}	(van den Heuvel et al., 1993)
wg^{IL114}	(van den Heuvel et al., 1993)
wg^{CX3}	(van den Heuvel et al., 1993)

wg^{P}	(van den Heuvel et al., 1993)
wg^{1}	(van den Heuvel et al., 1993)
P-element generated *wg* alleles	
wg^{r0727}	(U. Gaul; Neumann and Cohen, 1996a; This study)
Sp^{revP}	(Neumann and Cohen, 1996c; This study)
$Df(2L)spd^{j2}$	(Neumann and Cohen, 1996a)
$Df(2L)spd^{hL2}$	(Neumann and Cohen, 1996a; This study)
vestigial (*vg*) alleles	
vg^{83b27}	(Williams et al., 1994)
vg^{83b27R}	(Williams et al., 1993)
Suppressor of Hairless (*Su(H)*) alleles	
$Su(H)^{SF8}$	(Lecourtois & Schwiesguth, 1995)
$Su(H)^{AR9}$	(Lecourtois and Schweisguth, 1995)
dishevelled (*dsh*) alleles	
dsh^{v26}	(Klingensmith et al., 1994; Yanagawa et al., 1995)
dsh^{VA153}	(Perrimon and Mahowald, 1987)
dsh^{75}	(Nordermeer et al., 1994)
armadillo (*arm*) alleles	
$arm^{H8.6}$	(Klingensmith et al., 1989; Orsulic and Peifer, 1996)

lacZ reporter genes	
wg-lacZ	(Kassis et al., 1992)
wg^{r0727}	see above
wg (*spd* enhancer)-lacZ	(Neumann and Cohen, 1996a; This study)
neuralized-lacZ (*A101*)	(Ghysen & O'Kane, 1989)
vg (D/V boundary enhancer)-*lacZ*	(Williams et al., 1994)
vg ("quadrant enhancer")-*lacZ*	(Kim et al., 1996)
cut (D/V boundary enhancer)-*lacZ*	(Jack et al., 1991)

2.8: Materials

2.8.1: Chemicals

Bacterial media were purchased from Gibco-BRL, USA. Electrophoresis grade agarose was from FMC. Deoxyribonucleoside triphosphates (dNTPs) were purchased from Boehringer Mannheim, Germany. Glycerol and phenol were from Merck, Germany. BrdU was from Sigma, Germany. Other organic and inorganic chemicals were purchased from Sigma, Merck or Boehringer Mannheim. All protocols used double distilled deionised water (dd H_2O) throughout.

2.8.2: Radioactive isotopes

^{32}P-α-dCTP (3000 Ci/mmol) was obtained from DuPont, USA.

2.8.3: Enzymes

Restriction enzymes were obtained from Boehringer Mannheim, Germany, New England Biolabs, USA, or Pharmacia, Sweden. Bacteriophage T4 DNA ligase, calf intestinal phosphatase (CIP) and *E. coli* DNA polymerase I large fragment

(Klenow) were from New England Biolabs, USA. T7 RNA polymerase were purchased from Boehringer Mannheim, Germany.

2.8.4: Buffers and solutions

PBS: 140 mM NaCl, 7 mM Na_2HPO_4, 3 mM KH_2PO_4

PBX2: PBS + 0.2% Triton X100

PBT2: PBS + 0.2% Tween 20

modified Carnoy's: ethanol:acetic acid (3:1)

E-hyb (in situ hybrridization buffer): 50% Formamide, 5 x SSC, 100 µg/ml denatured salmon sperm DNA, 50 µg/ml heparin, 0.1% Tween 20

AP (Alkaline Phosphatase) buffer: 0.1 M Tris (pH 9.5), 0.1M NaCl, 0.05 M $MgCl_2$ (just before use, add 0.1% Tween 20)

HM (Homogenizing) buffer: 0.1 M Tris-HCl (pH 9), 0.1 M EDTA, 1% SDS

Mounting Medium: 80% Glycerol, 1 x PBS

2.8.5: Other materials

Kodak XAR5 X-ray film was purchased from Eastman Kodak, USA. Glycogen was purchased from Boehringer Mannheim, Germany. Phenol/chloroform/isoamyl alcohol mixture (25/24/1; v/v/v) was obtained from Amresco, USA.

CHAPTER 3:
THE MOLECULAR AND GENETIC CHARACTERIZATION OF TWO REGULATORY MUATIONS OF WINGLESS THAT AFFECT LARVAL DEVELOPMENT

CHAPTER 3: THE MOLECULAR AND GENETIC CHARACTERIZATION OF TWO REGULATORY MUATIONS OF WINGLESS THAT AFFECT LARVAL DEVELOPMENT

3.1: Introduction

As discussed in section 1.5 above, Wingless has many functions during Drosophila development. Since strong *wingless* mutants cause embryonic lethality, it is difficult to examine late functions of Wingless This has been done using the conditional *wingless*^{ts} mutant, or by making clones for strong *wingless* alleles in the larva. Another useful tool in studying late functions of *wingless* are regulatory mutants that specifically affect expression in the larva (*eg. wingless*^{1}; see Baker, 1988; Morata & Lawrence, 1977). In this chapter, I present the characterization of two regulatory mutations that affect some of the larval expression domains of *wingless*. These are *spade*^{flag}, which affects some of the late functions of *wingless* in the wing imaginal disc, and *Sternopleural*, which affects *wingless* functions both in the wing disc and in other discs.

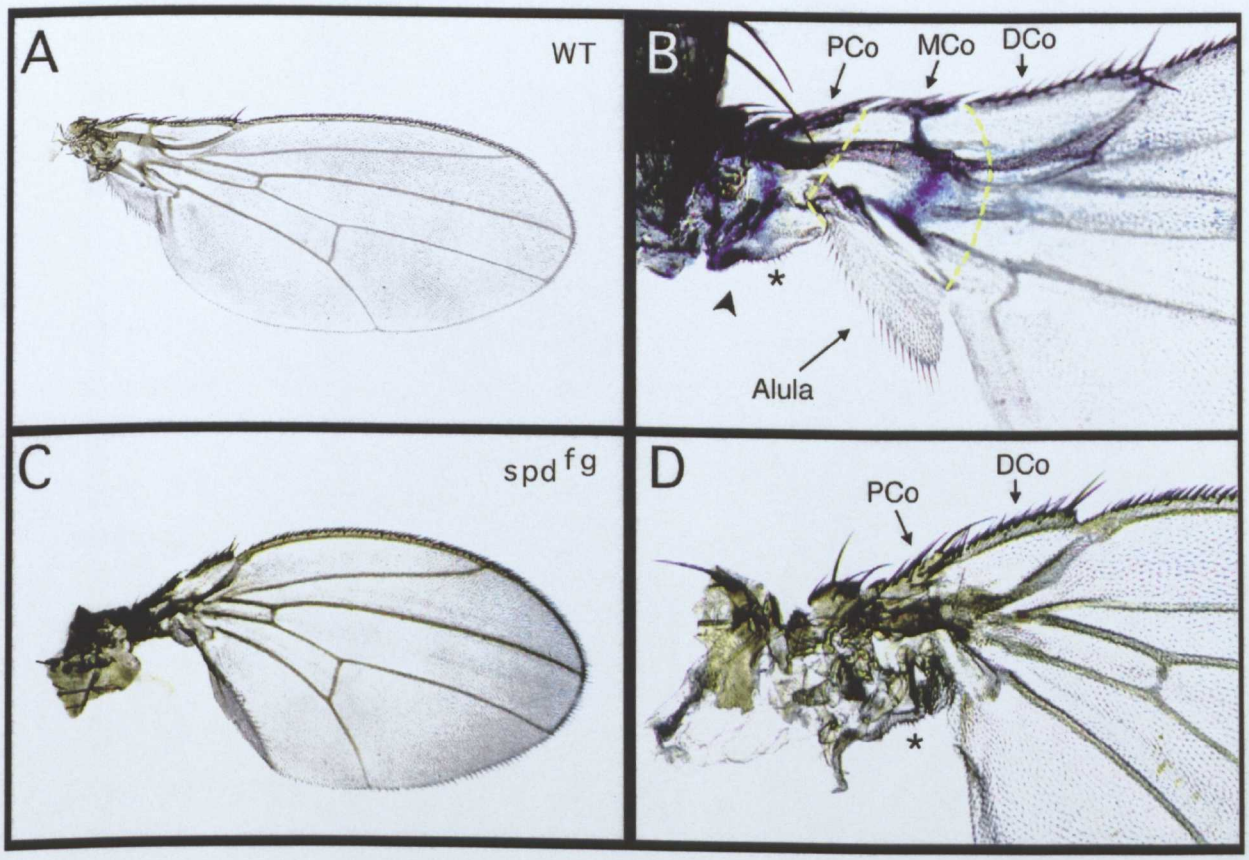
3.2: *spade*^{flag}

3.2.1: The mutation *spade*^{flag} reduces *wingless* expression in the wing hinge and at the D/V boundary of the wing

spade^{flag} (*spdfg*) was originally identified as a viable spontaneous mutation that causes an overall reduction of wing size, as well as a reduction of the alula and a variable loss of posterior wing margin structures (Lindsley and Zimm, 1992). A detailed characterization of the *spdfg* phenotype indicates that the most penetrant aspect of this phenotype is the loss of specific structures of the wing hinge (Fig.3.2.1).

Figure 3.2.1: The *spadeflag* mutant phenotype.

(A) Wild-type wing. (B) Close up view of the wild-type wing hinge from an individual carrying a *wg-lacZ* reporter. *wg*-expressing cells are stained blue. The proximal ring of *wg* expression (which corresponds to the outer ring in the wing imaginal disc) runs from the base of the proximal costa (PCo) to the proximal axillary cord (asterisk) adjacent to the body-wall (arrowhead). The more distal ring (which corresponds to the inner ring in the disc) runs from the center of the medial costa (MCo) to the base of the alula. (C) Wing from a fly homozygous for the mutation *spdfg*. The wing hinge is strongly reduced and the wing shows a slight overall reduction in size. There is a variable loss of wing margin bristles, which is stronger in the posterior margin. (D) Close up view of the *spdfg* hinge. Distal hinge structures including the medial costa (MCo), the humeral cross-vein, septum 2 and alula are missing. Proximal hinge structures, such as the proximal costa (PCo) and axillary cord (asterisk) are not affected. The hinge structures deleted in *spdfg* mutants are contained in the region bounded by the yellow dashed lines on the wild type wing in panel B. Note that the inner ring of *wg* expression lies in the center of this domain.

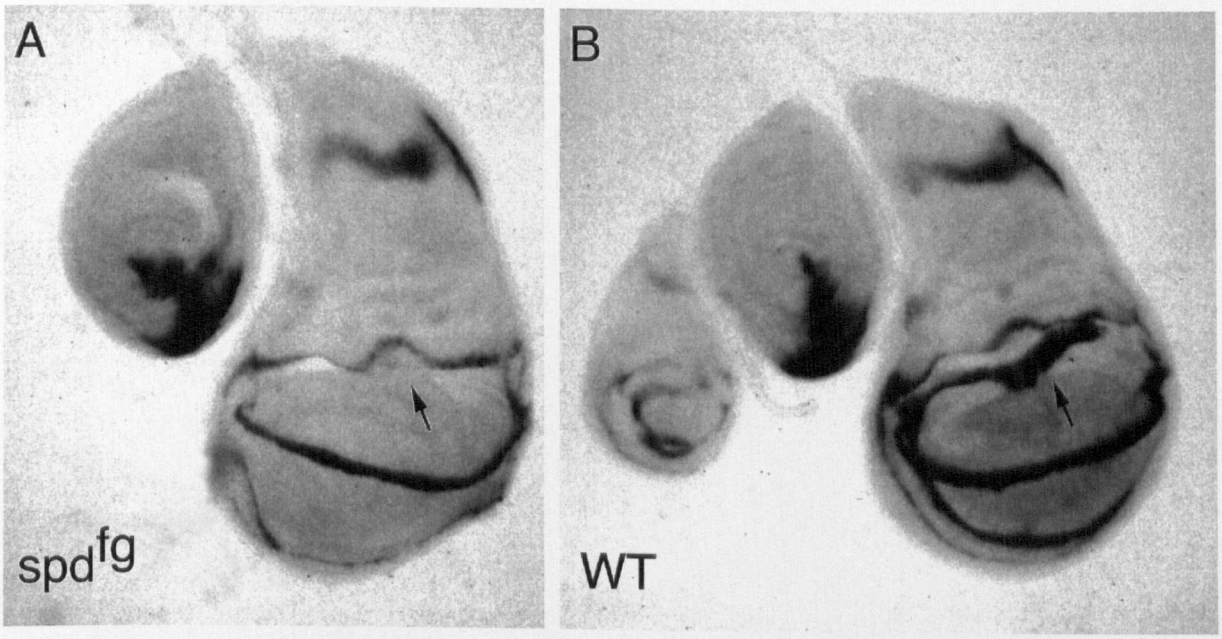

A
WT
B
PCo
MCo
DCo
Alula
C
spd fg
D
PCo
DCo

The hinge structures lost in *spdfg* include the medial costa, the humeral cross-vein, septum 2 and the part of the dorsal radius which lies between the medial costa and the alula (Fig. 3.2.1B,D). The alula is sometimes completely absent and sometimes vestigial. The Sc1 campaniform sensillum is always absent, while the Sc12 group of campaniform sensillae is reduced, with more proximally located sensillae sometimes remaining. More proximal hinge strucures, such as the proximal costa and the axillary cord are not affected by *spdfg*.

The stuctures lost in the hinge of *spdfg* mutants are centered on a domain of *wingless* (*wg*) expression that runs through the hinge (Fig. 3.2.1B,D). As discussed above (under section 1.5.2), *wg* is expressed in several domains in the third instar wing imaginal disc, including two rings surrounding the wing pouch (Fig. 3.2.2B). Examination of *wg-lacZ* expression in the adult wing (see Materials and Methods) shows that both of these rings of *wg* expression run through the hinge structures of the adult. The inner of the two rings in the imaginal disc corresponds to the more distal stripe running through the adult hinge. I will henceforth always refer to this as the 'inner ring'. The location of the two rings of *wg* expression with respect to adult structures has been previously described in Phillips and Whittle (1993). The inner ring runs from the medial costa through the humeral cross-vein and the dorsal radius and then curves around to the base of the alula (Fig. 3.2.1B). The outer ring runs from the base of the costa to the base of the axillary cord (Fig. 3.2.1B). The inner ring of *wg* expression lies in the hinge domain which is deleted in *spdfg* mutants. This raises the possibility that *spdfg* may reduce *wg* function associated with the inner ring of *wg* expression in the wing imaginal disc.

Figure 3.2.2: The inner ring of *wg* expression is lost in *spade*^{flag} mutants.

(A) Third instar wing imaginal disc from a *spdfg* mutant stained for *wg* RNA by *in situ* hybridization. Note that all domains of *wg* expression are present, except the inner ring surrounding the wing pouch (arrow). This ring corresponds to the distal ring of the adult wing hinge. Careful comparison of (A) and (B) also indicates that there is a slight reduction in the level of *wg* expression in the wing margin. (B) Wild-type third instar wing imaginal disc stained as in (A).


A
spd^fg
B
WT

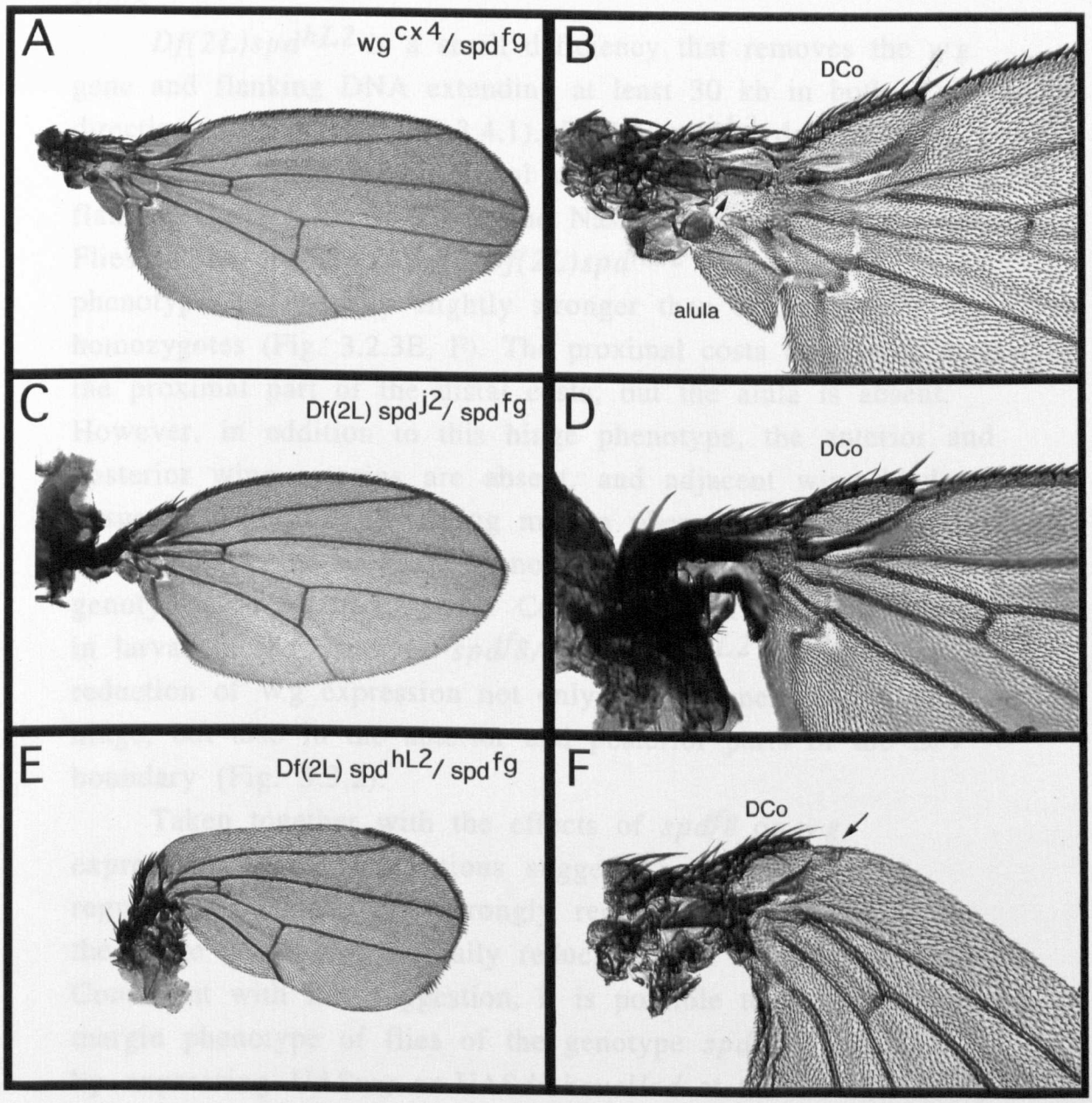
To test this possibility directly, I examined *wg* expression in *spdfg* mutant imaginal discs by whole-mount *in situ* hybridization. The inner ring of *wg* expression is not detectable in *spdfg* mutant discs (Fig. 3.2.2A). Careful examination also suggests that the intensity of staining at the D/V boundary is reduced relative to wild-type controls (compare Fig. 3.2.2A and B). This observation is consistent with a report by Couso et al. (1994). These authors noted that *spdfg* homozygotes show some loss of wing margin bristles and have a reduced expression of *wg* at the posterior D/V boundary.

The observation that *spdfg* removes *wg* expression in the inner ring of the wing hinge and reduces it at the D/V boundary is consistent with the proposal that *spdfg* may be a regulatory allele of *wg* (Tiong and Nash, 1990; Couso et al., 1994). The meiotic map location of *spdfg* is very close to *wg* (Lindsley and Zimm, 1992) and *spdfg* is included in all small deficiencies that remove the *wg* locus ((Tiong & Nash 1990)). To further explore this issue, I crossed *spdfg* to three well-characterized *wg* alleles: a *wg* null point mutant (wg^{CX4}), a deficiency removing only the 5' region of *wg* ($Df(2L)spd^{J2}$), and a deficiency removing both the 5' and 3' regions of *wg* ($Df(2L)spd^{hL2}$). For a full description of these three *wg* mutants see Figs. 3.2.7, 3.2.8, and 3.4.1 below.

Flies of the genotype $spdfg/wg^{CX4}$ show a milder version of the hinge phenotype seen in *spdfg* homozygotes (Fig. 3.2.3A, B) and there are no reductions of margin structures. If *spdfg* and *wg* were different loci that show a dominant genetic interaction, one would expect that a deficiency removing both would show the same phenotype as the $spdfg/wg^{CX4}$ heterozygous combination. However, deficiencies that uncover the whole region do not show any dominant phenotype, suggesting that *spdfg* is allelic to to *wg* (see also below).

Figure 3.2.3: *spade*^{flag} behaves genetically as a regulatory allele of *wg*.

(A,B) Wing from a fly heterozygous for *spd*^{fg} and *wg*^{CX4}. The hinge shows a mutant phenotype milder than that observed in the *spd*^{fg} homozygous wing. The alula and septum 2 (arrow) are present, but the medial costa and humeral cross-vein are missing. (C) Wing from a fly heterozygous for *spd*^{fg} and Df(2L)*spd*^{j2}. (D) Detail of the hinge region of a comparable wing. This phenotype is indistinguishable from that of *spd*^{fg} homozygotes, suggesting that Df(2L)*spd*^{j2} completely removes *spd* function.
(E, F) Wing from a fly heterozygous for *spd*^{fg} and Df(2L)*spd*^{hL2}. Note the extensive scalloping of the anterior and posterior wing margin. The wing is smaller than in *spd*^{fg} homozygotes (compare with fig 1C and 3C, which is similar in size to the wing of a *spd*^{fg} homozygote). (F) The hinge phenotype is slightly stronger than in the *spd*^{fg} homozygote. Arrow indicates where vein 1 reaches the anterior margin. Loss of wing margin structures begins here and extends distally. PCo and DCo are unaffected.


A
wg cx4 / spd fg
B
DCo
alula
C
Df(2L) spd j2 / spd fg
D
DCo
E
Df(2L) spd hL2 / spd fg
F
DCo

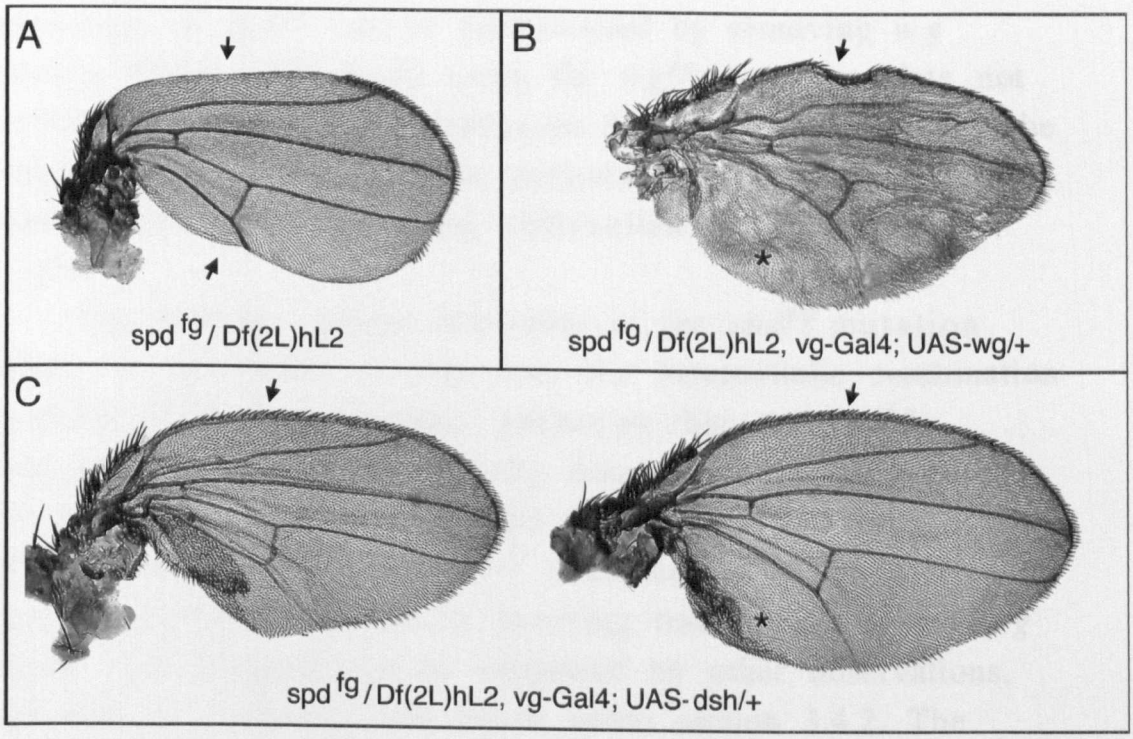
Df(2L)spd^{J2} removes the entire 5' regulatory region of *wg*, but leaves the promoter and coding region intact (see Fig. 3.4.1). When heterozygous with *spdfg*, *Df(2L)spd^{J2}* produces a phenotype that is indistinguishable from that of *spdfg* homozygotes (Fig. 3.2.3C, D). This shows that removing the 5' regulatory region of *wg* abolishes the ability to complement *spdfg*.

Df(2L)spd^{hL2} is a small deficiency that removes the *wg* gene and flanking DNA extending at least 30 kb in both directions (Figs. 3.2.8 and 3.4.1). *Df(2L)spd^{hL2}* does not include the other known lethal complementation groups flanking the *wg* locus (Tiong and Nash, 1990; data not shown). Flies of the genotype *spdfg/Df(2L)spd^{hL2}* have a hinge phenotype that is only slightly stronger than that of *spdfg* homozygotes (Fig. 3.2.3E, F). The proximal costa is present, as is the proximal part of the distal costa, but the alula is absent. However, in addition to this hinge phenotype, the anterior and posterior wing margins are absent, and adjacent wing blade tissue is scalloped. This wing margin phenotype is much stronger than that of *spdfg* homozygotes and of flies of the genotype *spdfg/Df(2L)spd^{J2}*. Consistent with this observation, in larvae of the genotype *spdfg/Df(2L)spd^{hL2}* there is a strong reduction of Wg expression not only in the inner ring of the hinge, but also in the anterior and posterior parts of the D/V boundary (Fig. 5.3.2).

Taken together with the effects of *spdfg* on *wg* expression, these observations suggest that *spdfg* is a regulatory mutation that strongly reduces *wg* expressiom in the hinge while only partially reducing it in the wing margin. Consistent with this suggestion, it is possible to rescue the wing margin phenotype of flies of the genotype *spdfg/Df(2L)spd^{hL2}* by expressing UAS*wg* or UAS*dishevelled* at the D/V boundary under the control of vestigial(intron2)GAL4 (vgGAL4; Fig. 3.2.4).

Figure 3.2.4: Rescue of the *spdfg/Df(2L) hL2* wing phenotype by *wg* activity.

(A) *spdfg/Df(2L) hL2* mutant wing. Note the loss of anterior and posterior wing margin, as well as the deletion of wing hinge structures typical of the *spdfg* homozygote wing.
(B) Partial rescue of the *spdfg/Df(2L) hL2* mutant phenotype by expression of *wg* cDNA under control of the early vestigial enhancer (vg-Gal4; UASwg). Note the rescue of the anterior wing margin (arrow) and of the posterior wing blade, between vein 5 and the posterior margin (asterisk). Because the combination of vg-Gal4: UASwg is pupal lethal, the wings were inflated from dissected pupae. The resulting wings appear less flattened and the veins are not as well defined.
(C) Partial rescue of the *spdfg/Df(2L) hL2* mutant phenotype by expression of *dsh* cDNA under control of the early vestigial enhancer (vg-Gal4; UASdsh). Note that expression of vg-Gal4 is significantly broader in the anterior and posterior wing margin regions than in the more distal wing margin (see Simmonds et al 1995). Therefore the relatively extensive rescue of the lateral regions of the hinge may reflect a low level of vg-GAL4:UASdsh in these cells. Note that the vg-GAL4 driver is not expressed in the central region of the wing hinge and so cannot rescue the *spdfg* hinge phenotype.

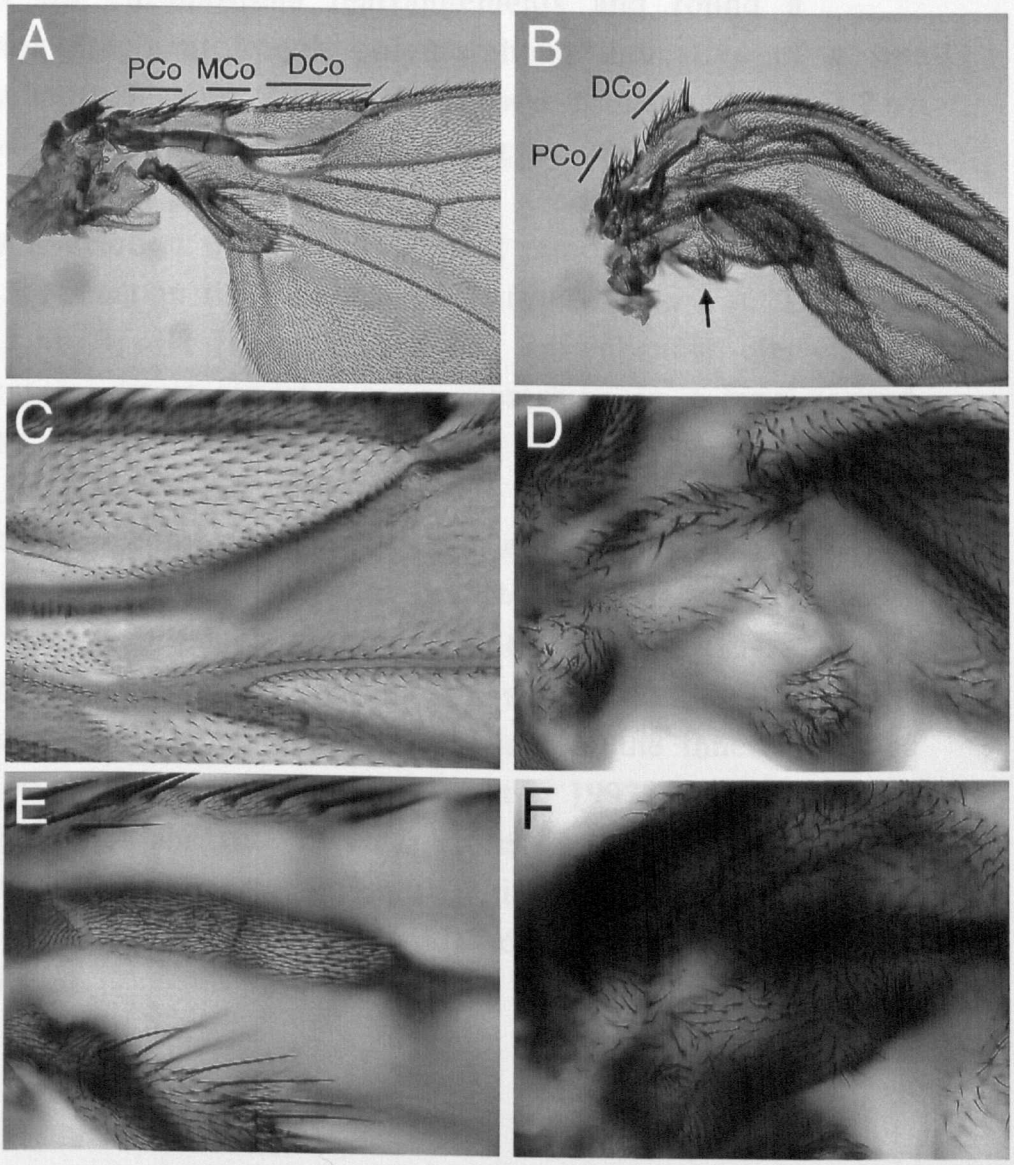

A
spd fg / Df(2L)hL2
B
spd fg / Df(2L)hL2, vg-Gal4; UAS-wg/+
C
spd fg / Df(2L)hL2, vg-Gal4; UAS- dsh/+

The vestigial intron 2 enhancer is expressed in the same cells at the D/V boundary as *wg* (Williams et al., 1994; Blair, 1994). In the vgGAL4 line (described in Simmonds et al., 1995), this enhancer is linked to a GAL4 cDNA, and drives the expression of GAL4 protein at the D/V boundary. This can be used to activate genes that have a UAS sequence linked to their promoter at the D/V boundary (for a full description of the GAL4:UAS technique, see the Materials and Methods section, and Brand and Perrimon, 1994). The ability of vgGAL4:UAS*wg* to rescue the wing margin phenotype of *spdfg* strongly indicates that this phenotype is due to a reduction of *wg* expression at the D/V boundary. Furthermore, the hinge phenotype of *spdfg* can be phenocopied by removing *wg* activity during third instar using the wg^{ts} mutation (data not shown), by large clones homozygous for wg^{CX4} that occupy the hinge area (F. Diaz-Benjumea, personal communication), or by clones homozygous for strong *dishevelled* (*dsh*) alleles (Fig. 3.2.5).

The complex genetic behaviour of the *spdfg* mutation raises two questions: 1) why does the heteroallelic combination $spdfg/wg^{CX4}$ have a weaker phenotype than the *spdfg* homozygous combination? 2) why does $Df(2L)spd^{J2}$ uncover the full hinge phenotype of *spdfg*, but not the full wing margin phenotype, while $Df(2L)spd^{hL2}$ uncovers both? The first question can be answered by invoking transvection at the *wg* locus. This proposal can be supported by other observations, and will be discussed fully below under section 3.4.2. The second question may be answered by invoking a partially redundant regulation of *wg* expression at the D/V boundary involving enhancer sequences located both 5' and 3' of the *wg* transcript. Further support for this issue will be discussed under section below 3.4.1.

Figure 3.2.5: Reduction of dishevelled function phenocopies the *spdfg* phenotype

(A, C, E) wildtype. (B, D, F) cuticle from individuals in which homozygous *dishevelled* (*dsh*) mutant clones occupy large patches of the hinge area. Note that large *dishevelled* clones that occupy the hinge generate a *spdfg* phenocopy (compare B with A, and also compare with Fig. 3.2.1). Although the *dsh* clones shown here were generated for analysis in discs (see Fig. 6.4.2), they could also be recognized in the adult cuticle because *dsh* mutant cells have a tissue polarity phenotype (eg. see Krasnow et al., 1995). In a wild-type wing, all hairs and bristles point towards the distal tip of the wing (C, E), while in *dsh* mutant tissue, hairs have random or reversed polarity (D, F). In all cases where a *spdfg* phenocopy was observed, large patches of *dsh* mutant tissue were found to occupy the hinge region (D is a close-up of the hinge shown in B, while F is a close-up of another hinge with a *spdfg* phenotype). pCo = proximal costa, mCo = medial costa, dCo = distal costa.


A
PCo MCo DCo
B
DCo
PCo
C
D
E
F

3.2.2: *spadeflag* deletes an enhancer element located 5' of *wingless* that drives expression in the wing hinge and at the D/V boundary of the wing

The observation that *Df(2L)spd*^{J2} uncovers the *spdfg* mutant phenotype suggested that *spdfg* must affect sequences located 5' of *wg*. I tested DNA in this region from *spdfg* mutants for chromosome rearrangements and found a restriction fragment length polymorphism indicative of a small deletion located about 9 kb 5' of the *wg* promoter (Fig. 3.2.6A-C). Comparison of the restriction enzyme digestion pattern of mutant genomic DNA with wild-type genomic DNA shows that there is a deletion of about 1 kb that removes most of a 1.2 kb *Eco*RI fragment in the wildtype DNA (probe c in Fig. 3.2.6C). This 1.2 kb *Eco*RI fragment contains an enhancer element sufficient to direct reporter gene expression in a ring around the wing pouch and in a stripe along the D/V boundary (Fig. 3.2.9). *lacZ* expression driven by this DNA fragment is activated in second instar in a diffuse pattern (Fig. 3.2.9A) which resolves into a stripe at the D/V boundary and a ring around the wing pouch during early third instar (Fig. 3.2.9B). The dynamics of this expression are similar to those of the endogenous *wg* gene in the margin and in the inner ring (see Couso et al., 1993; Phillips and Whittle, 1993). Staining of adult flies shows that the ring of *lacZ* expression corresponds to the inner ring of *wg* expression in the hinge, and that the stripe across the wing pouch corresponds to the wing margin (data not shown).

Figure 3.2.6: The mutation *spade^{flag}* is due to a small deletion in the 5' regulatory region of *wg*.

(A) An agarose gel of phage spd 1, digested with different enzymes, as indicated above the lanes. The fragments which hybridize to the 3.4 kb HindIII/BamHI fragment indicated in panel D as 'probe A' are marked by red arrowheads. (B) Genomic Southern blot of wild-type DNA, and DNA from *spdfg* homozygotes as indicated underneath the lanes. DNA was digested with BamHI or EcoRI (R1) as indicated. The blot was hybridized with a BamHI fragment from the *wg* 5' region that is indicated in panel D as 'probe B'. Note that the size of this Bam fragment is reduced in *spdfg* mutant DNA (compare the first two lanes), indicating that some DNA has been deleted. Comparison with the wild-type EcoRI digest shows that the small EcoRI fragment is gone in *spdfg* mutant DNA, and that a novel smaller fragment replaces the small BamHI/EcoRI fragment seen in the double digest of wild-type DNA, suggesting that one EcoRI site is lost in the mutant. This digestion pattern can be explained by a deletion which removes most of the small EcoRI fragment as well as the left EcoRI site (see the diagram in panel D). (C) The first two lanes of the blot shown in B were stripped and re-probed with the small EcoRI fragment shown in panel D as 'probe C'. This fragment labels the wild-type DNA strongly, but the *spdfg* DNA only very weakly (arrow), indicating that most of the DNA of this 1.2 kb EcoRI fragment is deleted in the mutant.
(D) Schematic map of the *wg* gene, showing the location of the deletion in *spdfg* (> <). The DNA fragments used to probe the genomic Southern blots in panels A, B and C are indicated on the map.
H = HindIII, B = BamHI, RI = EcoRI.

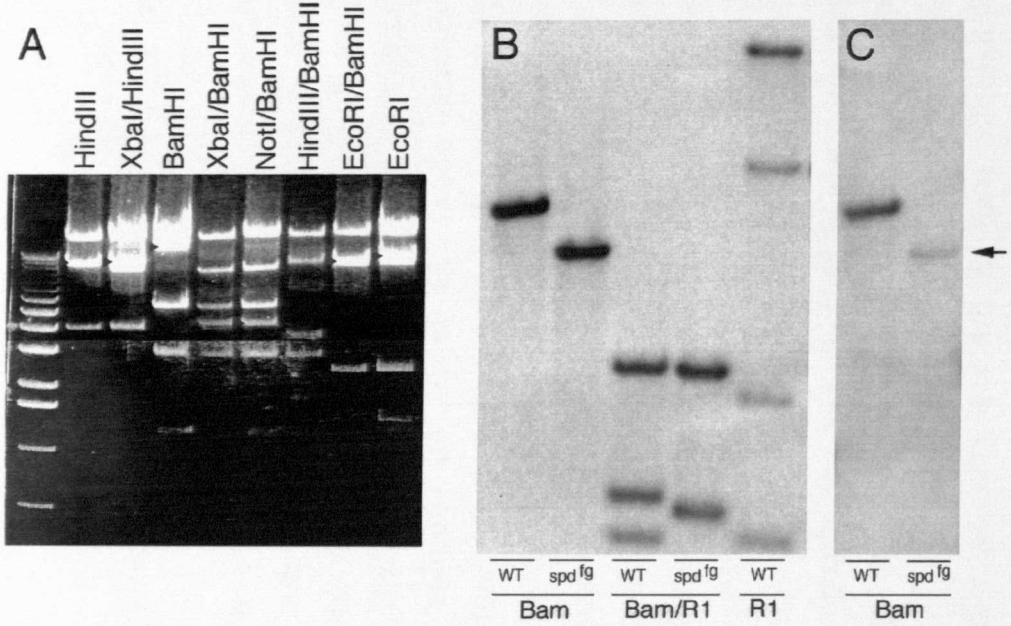

A
HindIII
XbaI/HindIII
BamHI
XbaI/BamHI
NotI/BamHI
HindIII/BamHI
EcoRI/BamHI
EcoRI
B
WT
spd fg
WT
spd fg
WT
Bam
Bam/R1
R1
C
WT
spd fg
Bam
D
probe B
probe C
wg
H B B B RI H RI B B H
spdfg
probe A
phage spd 1
1kb
Nusse wg clone 8

Figure 3.2.7: Insertion site of the *wg*^{r0727} P-element

(A) Agarose gel of the wg^{r0727} rescue plasmid, digested with different enzymes, as indicatedabove the lanes.
(B) Genomic Southern blot of wild-type DNA, and of DNA from individuals heterozygous for r0727, Df(2L)*spd*^{J2} and Df(2L)*spd*^{hL2}, as indicated above the lanes; all samples digested with HindIII/BamHI. The blot was probed with the 3.4 kb HindIII/BamHI fragment indicated in panel C as 'probe B". Note that (due to HindIII sites located at the ends of the PZ P-element) the r0727 insertion causes the 3.4 kb HindIII/BamHI fragment to be subdivided into two smaller fragments, the larger of which corresponds to the left flank (arrowhead), while the smaller fragment corresponds to the right flank. Note also that only the left flank is absent on the Df J2 chromosome, while both flanks are absent on the Df hL2 chromosome. This is consistent with the cytological data (Fig. 3.2.8; see also Fig. 3.4.1).
(C) Schematic map of the *wg* gene showing the relation of the r0727 insertion (triangle) to the *wg* transcript. The extent of the r0727 rescue flank as well as the probe used in panel B are indicated.
B = BamHI, H = HindIII, R = EcoRI.

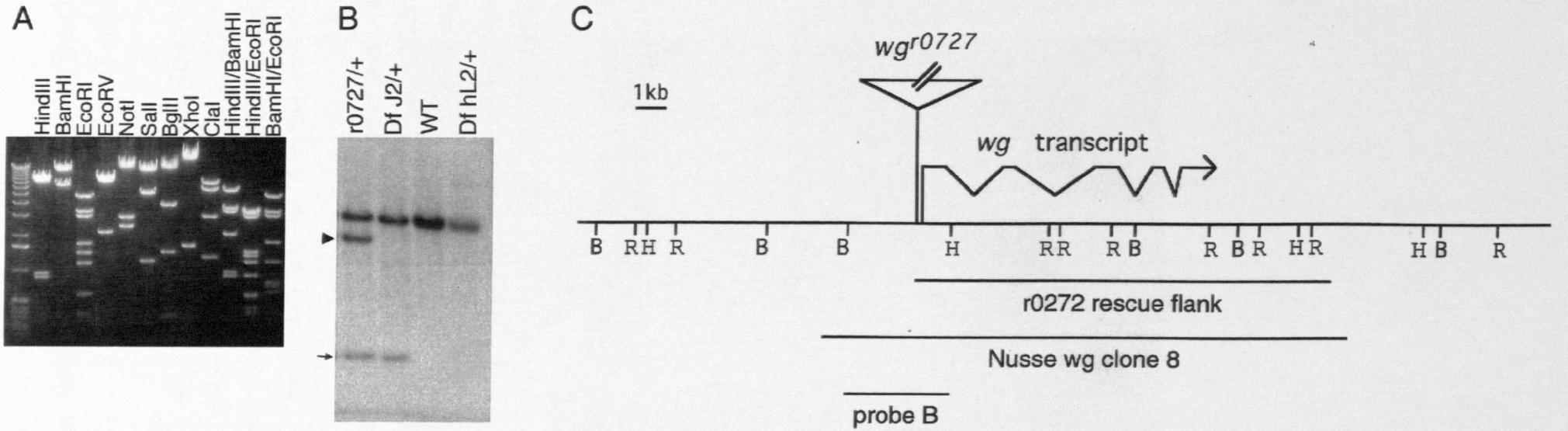

A
HindIII
BamHI
EcoRI
EcoRV
NotI
SalI
BglII
XhoI
ClaI
HindIII/BamHI
HindIII/EcoRI
BamHI/EcoRI
B
r0727/+
Df J2/+
WT
Df hL2/+
C
wg^r0727
1kb
wg transcript
B RH R B B H RR R B R B R HR H B R
r0272 rescue flank
Nusse wg clone 8
probe B

Figure 3.2.8: Cytological analysis of two deficiencies affecting the *wg* locus

(A) Polytene chromosome of an individual heterozygous for Df(2L)*spdj*^{2} hybridized with a *wg* cDNA probe (arrowhead). Although this deficiency leaves the *wg* coding region intact, it removes DNA 5' of *wg* extending from 28A1 to 27C1. See also Fig. 3.4.1.
(B) Polytene chromosome of an individual heterozygous for Df(2L)*spd*^{hL2} hybridized with a *wg* cDNA probe (arrowhead). Although this deficiency appears to be cytologically invisible, and hence must small, it removes the whole *wg* coding region, as no signal can be found to hybridize to the mutant chromatid (arrow).

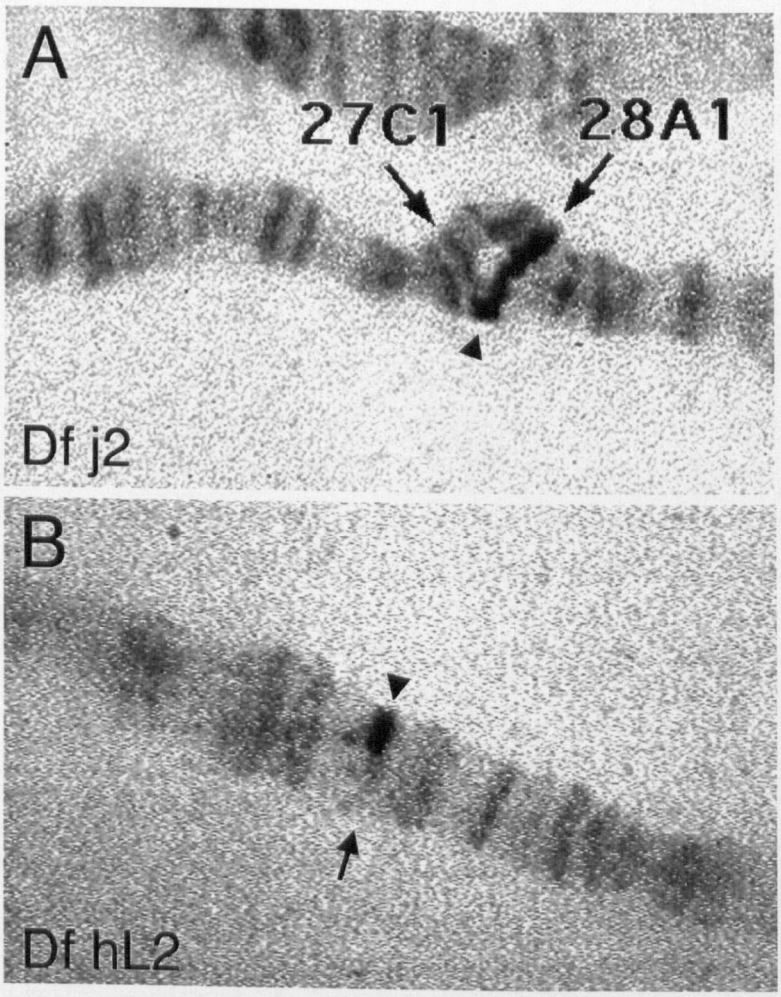

A
27C1
28A1
Df j2
B
Df hL2

Figure 3.2.9: The DNA fragment deleted in *spdfg* contains an enhancer element.

The 1.2 kb R1 fragment (described as probe C in Fig 3.2.6D) contains an enhancer element sufficient to drive *lacZ*-reporter gene expression in a pattern that corresponds to the inner ring and the wing margin expression domains of *wg*. All discs were stained with X-gal to reveal *lacZ* expression driven by the enhancer element. (A) A late second instar wing imaginal disc. Expression at this stage appears diffuse. (B) An early third instar wing disc showing an early stage in the formation of the wing margin stripe and a ring surrounding the wing pouch. (C) A mid third instar wing disc showing an essentially mature expression pattern. (D) A late third instar wing disc. Staining corresponds to the wing margin and the inner ring of wg expression (compare this disc to the one in Fig. 2B, which shows the *wg* expression at a comparable stage). Note that expression in the more distal regions of the wing margin is a bit broader than that of endogenous *wg*, and that staining of the ventral part of the ring is not as strong as of *wg* in this domain (this is especially apparent in B and C).

A
B
C
D

Figure 3.2.10: Sequence of the *spd* enhancer

The sequence of the 1.2 kb EcoRI fragment indicated as 'probe C' in panel D of Figure 3.2.6. The bases contibuting to a potential Suppressor of Hairless binding site are underlined. Note that this site does not conform fully with the consensus. The consensus for Su(H) sites is: T/CGTGA/GGAAA/C (Bailey and Posakony, 1996; Lecourtois and Schweisguth, 1996). All Su(H) binding sites identified so far fully conform with this sequence, making it doubtful that the sequence underlined here (which does not conform at positions 6 and 9) is functional.

1 ACGGTATCGA TAAGCTTGAT ATCGAATTCT CGTGAACCTC CCAGCACATC

TTACTCATCC ATTTAATTGC AATTTGATTG AATTTGCAGA TTTTGTACAC

101 ATCATTCGAG TGGCTCCCAT GGGTCCATTT CGATCTGCTT CCAAAAATCC

ATTAGATGAA TCAAATCGAA TCGCAGGGAA GCAAACAAAC CGCGGAAAAA

201 ATGAATGTCG CTACAGAATG TTTAACCCTC GTTAAAATGA ATGAAAGCTT

ATTAAAAATA TTTCTTTATT ATTGAACAAA AAAATGGTCG ACCGAGAAAC

301 AAAAGAAACG TTTTTTTATT GTCATGGGCT TACGCGAAAA AATCACGAAA

AGCTAAGCAA AAATTAATAA ATAAAAAACA GGCGTACAAA TGTTAAACAT

401 TTGCCACGAT GCCATGACGA GAGTTGACTG TCCAAAAAGG ATCGAAGTTA

GTTGAATTGA ACACCGACTG ACTGACAGCT CGGGATCTGC CCATGAAGAT

501 AATGACGAAT CCGTCGCGTA ACAAGGATCT GCAATGCAAA ACCGTCTCAG

AAATCCTTAA CGAAAGAAAT AAATCTCCGA GGATCGTTCT AGTTTTTTGC

601 TGGCCAGCAC AAAAATGTCT CCGGAGAATT GCACGCTCTC GGAGATGAAA

TGTCGAAGAA AAAGTCTGCA AAAAAATCCA AAAACCAAAT GAAATGCGTA

701 CACAAAAATG CCAAAAAGCA GCAGAAGCCA CAGCAGAAGA GACAGAATTT

TTTGCCAACT TTTTGTCGAT GCGAAACTGT CACAGGAAAA AGGATTTTTG

801 GTTCGAGATT GGTTTTTTCT CTGCCTCTTT GCTGCTTCAC ATGGCATATT

TAAAGTTATG GGCCTCCGTC TTAGCCATTT AAAACGTTTG CGAAATTAAT

901 ATTGGCAACA AAAACTGTGA AATGC<u>TGTGA AAAG</u>AACGCG CGGCCAGCAG

TAGAGGTCCG ATGACTTCTC TTCGGCCAGC AAAAAGTAAT TTTTGCTAAA

1001 TCGCAAAAAA ATCAAACAAG ATCATGATCG GACGGATGAC GATAATGATG

GCCATGGTCA TGATGGCCAG GCAGTCGGAC AAGCGAATGA CGAATAGCCC

1101 ATAGCTCTGC GGCTCGGAAC GAATGCGAAA TTTACGGAAT CCGCCAAGAC

CCAAACCAGC AATTCCAAGC AATTCCCCAC AACGACAATT AAGCACGAAC

1201 AAAAGGCGAG CCACGGCGAG AAGATCGTGT TTGGAGTATC TGGAAGATCG GG

These results suggest that the *spdfg* mutant phenotype is due to the deletion of an enhancer element that drives *wg* expression in the inner ring of the wing hinge and in the wing margin. This is consistent with the genetic and phenotypic data presented in the previous section. Although the 1.2 kb *Eco*RI fragment drives strong expression at the D/V boundary, removal of this fragment in *spdfg* homozygotes does not cause a severe loss of wing margin structures. This may be due to a partial redundancy in this aspect of *wg* regulation, as already suggested above (see also section below 3.4.1).

3.3: *Sternopleural*

3.3.1: The mutation *Sternopleural* reduces *wingless* activity in the distal antenna, the anterior notum and at the D/V boundary of the wing

Sternopleural (*Sp*) was first identified as a dominant mutation that produces an increase in the number of sternopleural bristles (Lindsley and Zimm, 1992) (Fig. 3.3.1A, B). Homozygous *Sp* animals die at pupal stages before cuticle deposition. *Sp* has been mapped genetically to 2-22.0, and cytologically to the region 27C1-28C1 (Lindsley and Zimm, 1992). Within the limits of resolution of these techniques, this is the same chromosomal location as *wg*. I observed that an allele of *wg*, wg^P, also produces a weak *Sp*-like dominant phenotype (Fig. 3.3.1C), suggesting that *Sp* may affect the *wg* locus. Indeed, when *Sp* is crossed to the two pupal lethal *wg* alleles that have been mapped to the *wg* 3' regulatory region, wg^{CX3} and wg^P (Baker 1988a,b), the resulting individuals are pupal semi-lethal (Table 1). Crosses to other *wg* alleles are not lethal, suggesting that *Sp* may be a regulatory allele of *wg*.

Figure 3.3.1: The *Sp* dominant phenotype.

(A) A wild type cluster of sternopleural bristles, indicated by the bracket. Anterior is to the left and dorsal is up. (B) In *Sp*/+ mutants there is an increase in the number of sternopleural bristles, affecting both macrochaetae and microchaetae. (C) In wg^{P}/+ mutants a mild dominant *Sp*-like phenotype can be observed with about 20% penetrance.

A
wt
B
Sp/+
C
wg P/+

Figure 3.3.2: The *Sp* loss of function phenotype in the notum.

(A) Dorsal view of a part of the notum of a wild type individual carrying the *wg* lac-z reporter. The anterior dorsocentral bristle (aDC) lies on the edge of the stripe of *wg* expression in the notum (blue label). Anterior is to the left. (B) Individuals of the genotype Sp/wg^{P} frequently lack one or both anterior dorsocentral bristles. The position in which the aDC should be located is marked by the purple circle. (C) Individuals of the genotype Sp/wg^{CX4} also frequently lack the anterior dorsocentral bristles. There also appears to be a reduction in the number of micro-bristles located in the *wg*-expression domain, suggesting a stronger reduction in *wg* function in this combination.

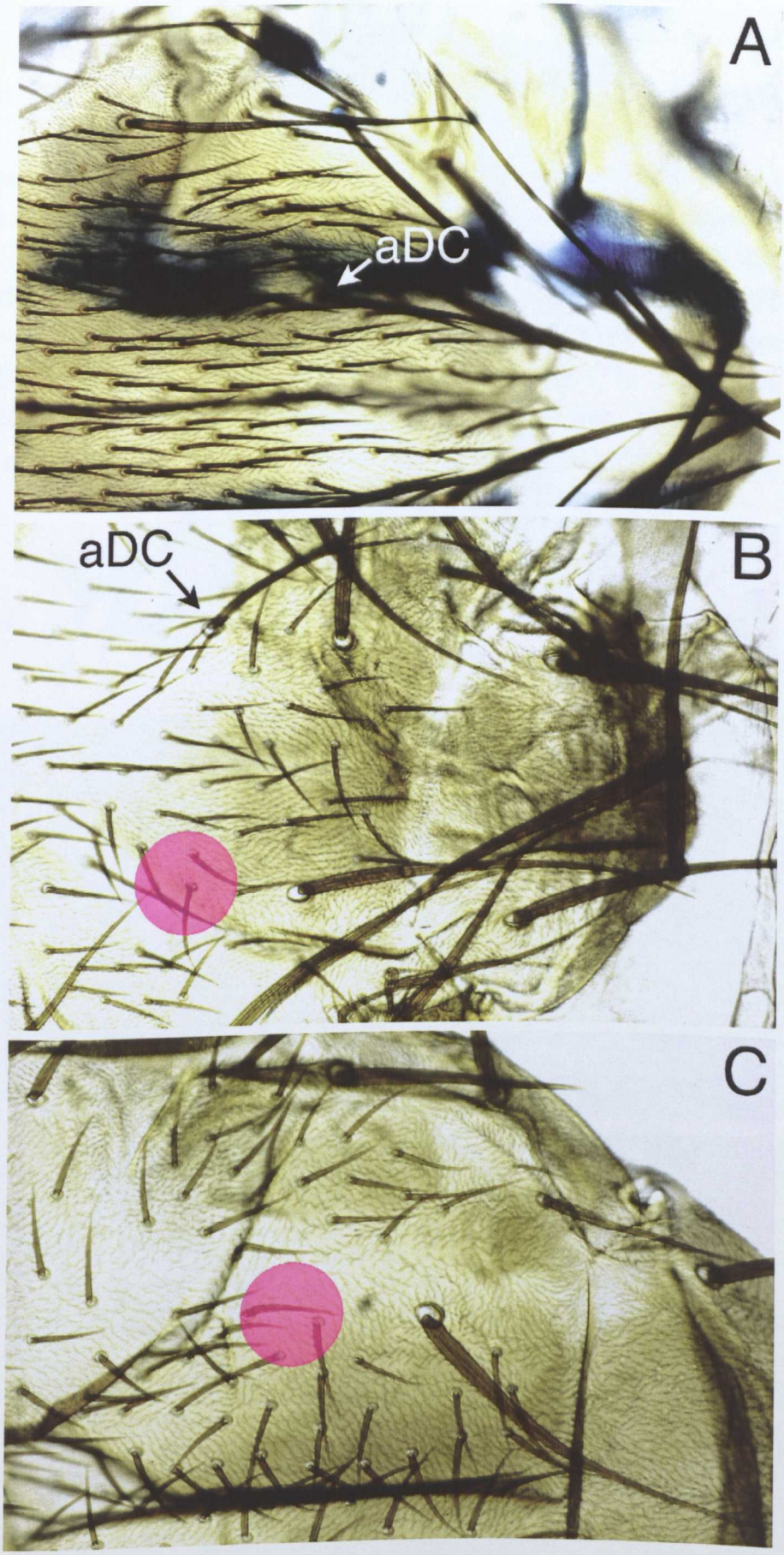

A
aDC
aDC
B
C

Figure 3.3.3: The *Sp* loss of function phenotype in the antenna.

(A) A wild type head showing the normal morphology of the antenna. There are three antennal segments. Segments AII and AIII are marked. The arista (ar) is a distal outgrowth of the AIII. (B) Pharate individuals of the genotype Sp/wg^{CX3} show phenotypes ranging from reduction of the arista (small arrow on the left side) to reduction of segment AIII (large arrow on the right). The phenotype is variable and incompletely penetrant. (C) Pharate individuals of the genotype Sp/wg^{P} show a loss or reduction of the arista (arrows).

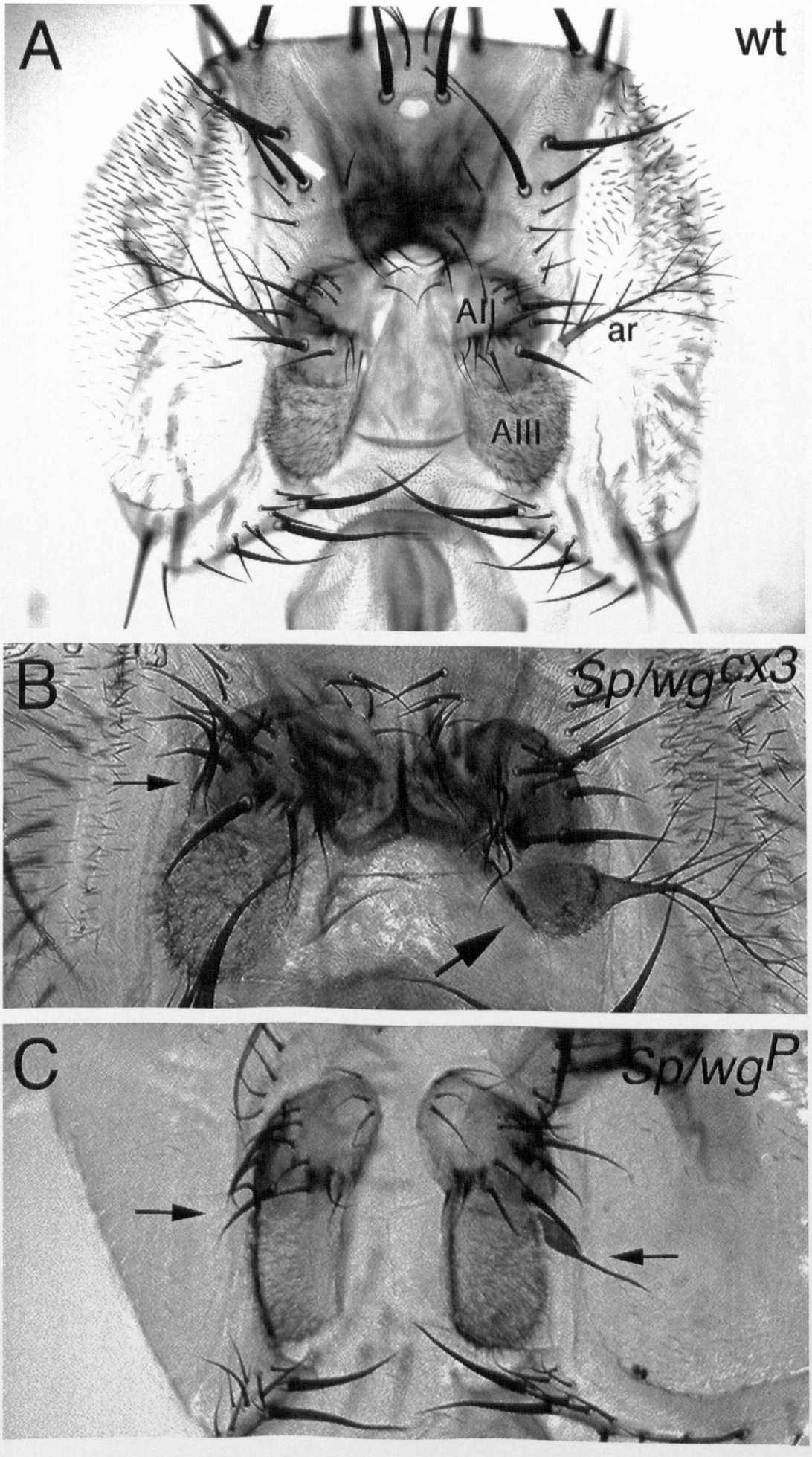

A
wt
AII
ar
AIII
B
Sp/wg^cx3
C
Sp/wg^P

Table 3.1: Complementation behaviour of several mutations at the *wg* locus

TABLE 1

Complementation behaviour of several mutations at the wg locus

	wg^{1}	*wg*^{P}	*wg*^{CX3}	*wg*^{CX4}	*Df(2L)spd j2*	*Sp revP*	*Sp*
Sp	wild type	semi-lethal pharates lack distal antenna & adc bristles	semi-lethal pharates lack distal antenna & adc bristles	adult viable lack adc bristles	adult viable loss of distal antennaand adc bristles	pupal lethal	pupal lethal
Sp revP	adult viable *wg*^{1}phenotype 100% penetrance	pupal lethal	pupal lethal	pupal lethal pharates lack antennae, legs and adc bristles	pupal lethal	pupal lethal	
Df(2L)spd j2	wild type	lethal before pupariation	semi-lethal escapers lack antennae & first leg pair	embryonic lethal	embryonic lethal		
wg^{CX4}	adult viable *wg*^{1}phenotype 15% penetrance	pupal lethal	pupal lethal	embryonic lethal			
wg^{CX3}	adult viable *wg*^{1}phenotype 35% penetrance	pupal lethal	pupal lethal				
wg^{P}	adult viable *wg*^{1}phenotype 80% penetrance	embryonic lethal*					
wg^{1}	adult viable *wg*^{1}phenotype 80% penetrance**						

* data from van den Heuvel et al., 1993
** data from Morata and Lawrence 1977

Examination of the few adult escapers of crosses between *Sp* and wg^{P} and wg^{CX3} reveals that they have two distinct phenotypes, each of which can be attributed to reduced *wg* activity. The escapers often lack one or both anterior dorsocentral bristles and more rarely also the posterior postalar bristle and the presutural bristle (Fig. 3.3.2 and Table 1). These three bristles are among the six macrobristles in the notum (or dorsal thorax) that have been shown to require *wg* function for their development (Phillips and Whittle, 1993). Removal of *wg* function during third instar using the wg^{ts} allele leads to loss of these bristles, which develop close to the *wg*-expression domain in the notum. I also noticed that in the allelic combinations *Sp*/wg^{CX3} and *Sp*/wg^{P}, microbristles located inside this *wg*-expression domain may also be lost (Fig. 3.3.2). The second phenotype is loss of distal antennal segments, and in very rare cases, antennal duplication (Fig. 3.3.3 and Table 1). This phenotype can also be obtained by shifting animals of the genotype wg^{ts}/wg^{CX3} to the nonpermissive temperature during late third instar; also, wg^{CX3} homozygous pharate indiviuals do not have any antennae at all (Baker, 1988).

These results suggest that *Sp* is a regulatory mutation of *wg* that compromises *wg* function in the antenna and the notum. This interpretation is supported by the observation that *wg* expression in the anterior notum is strongly reduced in late third instar wing imaginal discs of *Sp* homozygous larvae (Fig. 3.3.7) and in wing discs of larvae of the genotype *Sp*/*Df(2L)spd*^{hL2} (data not shown), as well as in wing discs of the genotype *spdfg Sp*/wg^{CX4} (Fig. 6.2.2). It is noteworthy that there is some Wg expression left in the posterior notum of these discs (Figs. 3.3.7 and 6.2.2), which correlates well with the observation that the more posteriorly located *wg*-sensitive bristles (the scutellar bristles and the posterior dorsocentral bristle) are not affected by *Sp*. A corresponding reduction of Wg expression in the antennal disc could not be found (data not shown), but as the antennal phenotype of *Sp* is relatively

weak when crossed to wg^{CX3} (which is a strong mutant for the *wg* antennal function, see Table 1), it may not be possible to visualize what could be a subtle reduction in *wg* expression.

Furthermore, the outer ring of Wg expression in the wing hinge (as opposed to the inner ring in *spdfg* mutants) is also absent in wing discs of larvae of the genotype $Sp/Df(2L)spd^{hL2}$ (data not shown) as well as in larvae of the genotype *spdfg* Sp/wg^{CX4} (Fig. 6.2.2). These discs also show a reduction in the level of Wg expression at the D/V boundary (Figs. 6.2.2 and 6.5.2). Unfortunately, individuals of the genotype $Sp/Df(2L)spd^{hL2}$ die as pupae before cuticle deposition, so it is not possible to examine the corresponding adult phenotype. However, taken together, the phenotypic and expression data suggest that *Sp* is a regulatory mutation of *wg* that reduces *wg* expression in the distal antenna, the anterior notum, the outer ring of the wing hinge, and at the D/V boundary of the wing. There are some viable allelic combinations of *wg* with *Sp* that cause wing margin scalloping (see next section), further supporting the proposal that *Sp* reduces *wg* function at the D/V boundary.

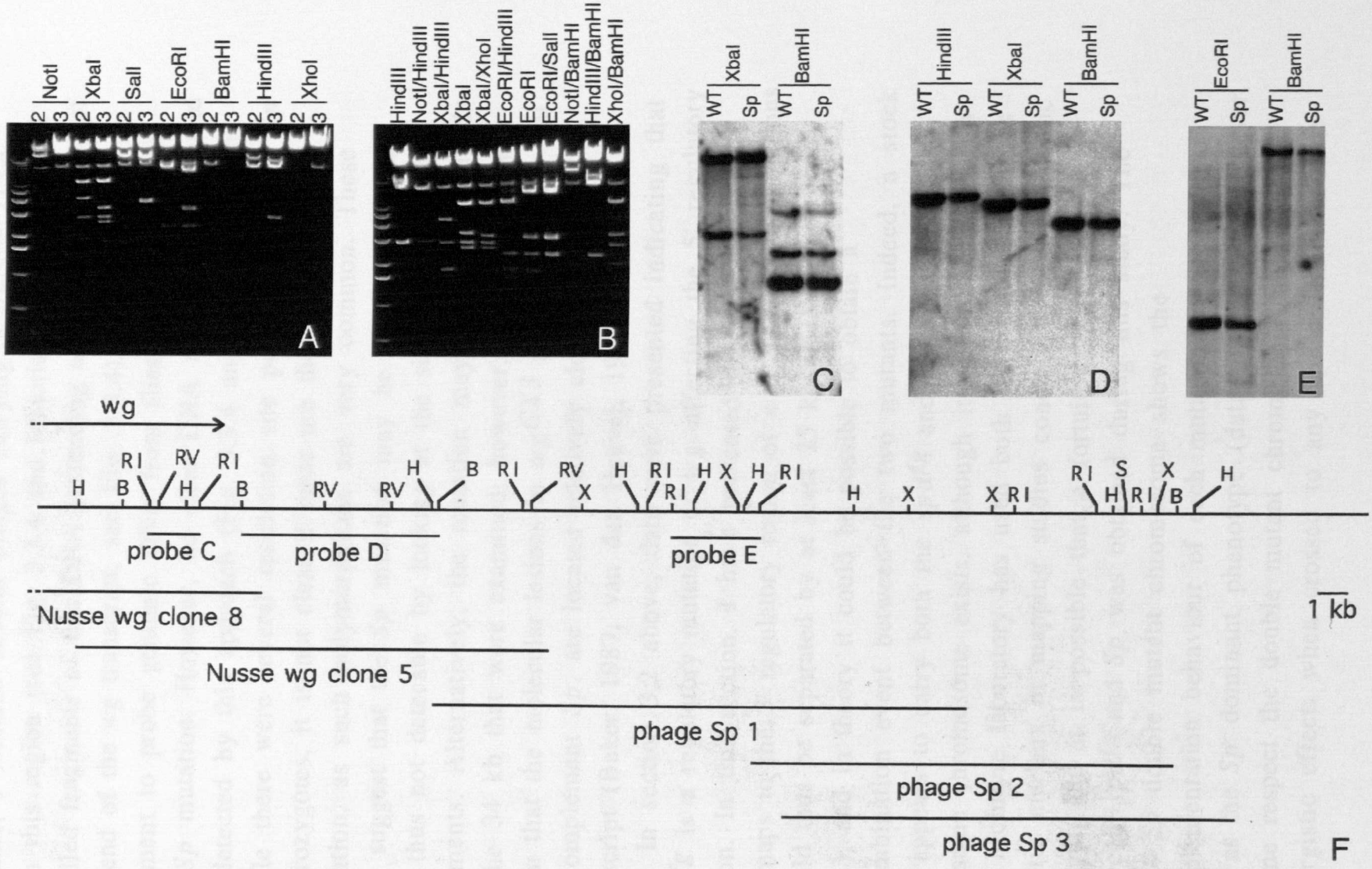
3.3.2: *Sternopleural* shows a comlex complementation behaviour with *wingless* alleles, and genetically maps to the 3' regulatory region of *wingless*

When *Sp* is crossed to wg^{CX4}, a *wg* null point mutant (van den Heuvel et al., 1993; see also Fig. 3.4.1), the resulting individuals are adult viable. However, they do show loss of anterior dorsocentrals and of microbristles located in the *wg*-expression domain of the notum (Fig. 3.3.2 and Table 1). $Df(2L)spd^{J2}$ is a large deficiency that removes all 5' sequences of *wg* but leaves the coding region intact (see Fig. 3.4.1). When crossed to this deficiency, *Sp* is adult viable, showing the same phenotype as over wg^{CX4}, with the addition that distal antennal segments are missing (Table 1). These two instances of complementation of the lethality of *Sp* by strong *wg* alleles may be due to transvection. This point will be discussed in more detail in section 3.4.2 below.

$Df(2L)spd^{hL2}$ is a small deficiency that removes the *wg* transcript and flanking DNA in both directions (see Fig. 3.4.1). When *Sp* is crossed to this deficiency, the resulting individuals are pupal lethal before cuticle deposition (at roughly the same stage when *Sp* homozygotes die). The observation that the lethality of *Sp* is complemented by $Df(2L)spd^{J2}$, which only removes the 5' region of *wg*, but not by $Df(2L)spd^{hL2}$, which removes both the 5' and 3' regions of *wg*, strongly suggests that the mutation *Sp* must affect the 3' region of *wg*. This is further supported by the observation that wg^{CX3} and wg^{P}, two regulatory mutations of *wg* that map to the 3' regulatory region, do not complement *Sp* (Table 1). Furthermore, wg^{P} also shows a weak *Sp*-like dominant phenotype, and the dominant phenotype of *Sp* can be reverted by the insertion of a P-element into the 3' regulatory region of *wg* (see section 3.3.3 below). These observations suggests that both the recessive and the dominant *Sp* phenotypes are due to a mutation in the 3' regulatory region of *wg*.

Figure 3.3.4: Characterization of the *wg* 3' UTR in *Sp* mutants

(A) Agarose gel of phages Sp 2 and Sp 3, digested with different enzymes, as indicated above the lanes. (B) Agarose gel of phage Sp 3, digested with different enzymes, as indicated above the lanes. (C) Genomic Southern blot of wild-type DNA, and of DNA from individuals heterozygous for the Sp mutation, digested with different enzymes, as indicated above the lanes. The blot was probed with the fragment indicated as 'probe C' in panel F. (D) Genomic Southern blot of wild-type DNA, and of DNA from individuals heterozygous for the Sp mutation, as indicated above the lanes. The blot was probed with the fragment indicated as 'probe D' in panel F. (E) Genomic Southern blot of wild-type DNA, and of DNA from individuals heterozygous for the Sp mutation, as indicated above the lanes. The blot was probed with the fragment indicated as 'probe E' in panel F. Note that in this blot, as in C and D, no polymorphisms can be detected on the Sp chromosome. In this panel, a large BamHI fragment, which encompasses nearly all of the cloned DNA to the right of 'probe D' (see panel F) appears to be of normal size in Sp/+ individuals (compare the two lanes at the right). (F) Schematic map of the wg 3' UTR. All the genomic clones obtained in this area, as well as the probes used for the blots in C, D and E, are indicated.
B = BamHI, H = HindIII, RI = EcoRI, RV = EcoRV, S = SalI, X = XbaI.


2 3 NotI
2 3 XbaI
2 3 SalI
2 3 EcoRI
2 3 BamHI
2 3 HindIII
2 3 XhoI
A
HindIII
NotI/HindIII
XbaI/HindIII
XbaI
XbaI/XhoI
EcoRI/HindIII
EcoRI
EcoRI/SalI
NotI/BamHI
HindIII/BamHI
XhoI/BamHI
B
WT Sp XbaI
WT Sp BamHI
C
WT Sp HindIII
WT Sp XbaI
WT Sp BamHI
D
WT Sp EcoRI
WT Sp BamHI
E
wg
H B RI H RV B RI
RV RV H B RI RV X H RI RI H X H RI H X X RI RI S H RI X B H
probe C
probe D
probe E
Nusse wg clone 8
Nusse wg clone 5
phage Sp 1
phage Sp 2
phage Sp 3
1 kb
F

To test for DNA rearrangements in the *wg* 3' region of *Sp* mutants, I isolated several phages carrying wild-type DNA from this region (see Fig. 3.3.4 and Materials and Methods). I labelled fragments of this DNA (extending as far as 34 kb 3' of the end of the *wg* transcript, see Fig. 3.3.4), and used these fragment to probe genomic DNA from flies heterozygous for the *Sp* mutation. However, no clear DNA rearrangements could be detected by this approach (Fig. 3.3.4 and data not shown). While there were several restriction site polymorphisms in *Sp* heterozygotes, it is not clear if these are the cause of the mutation, as such polymorphisms are very common. These data suggest that the *Sp* mutation may be relatively subtle, and thus not detecable by looking at the size of restriction fragments. Alternatively, the mutation may be located outside of the 34 kb that were examined; however, this is unlikely, given that the molecular lesions in wg^{CX3} and wg^{P}, which fail to complement *Sp*, are located relatively close to the *wg* transcript (Baker, 1987; van den Heuvel, 1993; Fig. 3.4.1).

In section 3.2 above, data were presented indicating that *spdfg* is a regulatory mutation of *wg* affecting the 5' regulatory region. In this section, I have presented data suggesting that *Sp* maps to the 3' regulatory region of *wg*. These two mutations should thus be separated by at least 25 kb of DNA (see Fig. 3.4.1), and in theory it could be possible to obtain a recombination event between the two mutants. Indeed, a stock that appears to carry both the *spdfg* and the *Sp* mutation on the same chromosome exists, although its origin is not known. The McIntyre laboratory has used both of these mutations as genetic markers in mapping studies conducted in this genomic interval, and it is possible that a fortuitous recombinant between *spdfg* and *Sp* was obtained during this study. The *spdfg Sp* double mutant chromosome shows the complementation behaviour of each mutation on its own, as well as the *Sp* dominant phenotype (data not shown). However, in one respect the double mutant chromosome has a synergistic effect: when crossed to any mutant that uncovers

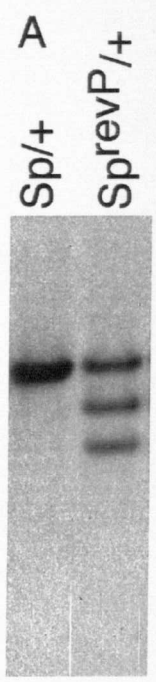
the wing margin phenotype of *wg*, the double mutant chromosome has a stronger phenotype than either mutation alone (*eg.* when crossed to wg^{CX4}, Fig. 6.5.2). This is consistent with the suggestion that both *spdfg* and *Sp* reduce *wg* function at the D/V boundary, *spdfg* by deleting a D/V boundary enhancer located 5' of *wg*, and *Sp* by reducing the activity of a D/V boundary enhancer located 3' of *wg*.

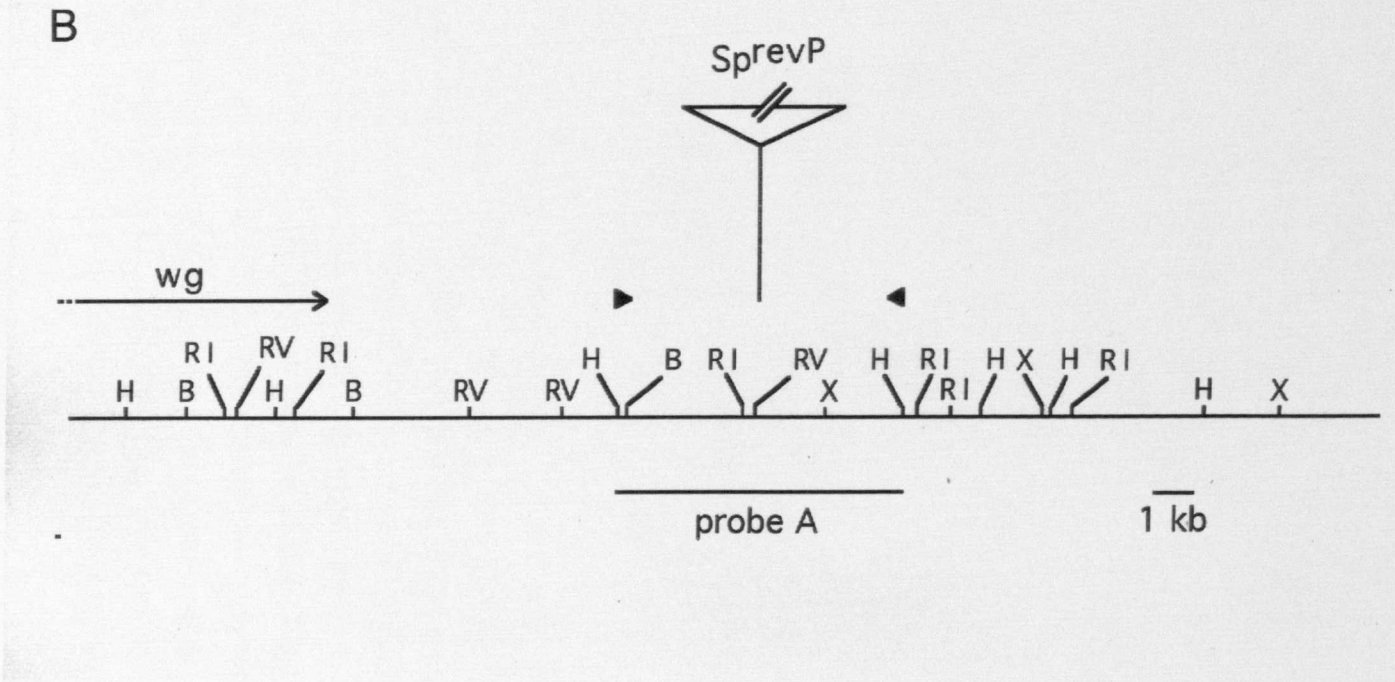
3.3.3: The *Sternopleural* dominant phenotype is likely to be due to Wingless misexpression in the dorsal first and second leg discs.

To identify the gene responsible for the *Sp* dominant phenotype, I attempted to revert this phenotype by P-element mutagenesis. The strategy for this screen is given in detail in the Materials and Methods section. Briefly, I crossed a chromosome carrying a P-element close to the *wg* gene to a marked *Sp* chromosome, together with a chromosome expressing a constitutive source of transposase, in the hope that a P-element might jump onto the *Sp* chromosome and thereby inactivate the gene responsible for the *Sp* dominant phenotype. One revertant of *Sp* was obtained in this way, and designated Sp^{revP}. In Sp^{revP} heterozygotes, the *Sp* dominant phenotype is almost completely suppressed; only 10% of these individuals show a mild *Sp* phenotype. While *Sp* is adult viable over wg^{CX4}, individuals of the genotype Sp^{revP}/wg^{CX4} die as pupae (Table 1), suggesting that *wg* activity is further reduced by the mutation reverting the *Sp* dominant phenotype. Consistent with this result, several ems-induced revertants of *Sp* fail to complement wg^{CX4} (M. Buratovich, personal communication). These results suggest that *wg* activity is necessary to generate the *Sp* dominant phenotype.

Figure 3.3.5: The *Sp*^{revP} P-element is inserted in the 3' region of the *wg* gene of the *Sp* chromosome

(A) Genomic Southern blot of DNA from individuals heterozygous for the Sp and Sp^{revP} mutations, as indicated above the lanes, digested with HindIII. The blot was probed with the 4 kb HindIII fragment indicated as 'probe A' in panel B. Note that this DNA fragment is cut in two in Sp^{revP}/+ individuals, suggesting that the P-element has inserted into this fragment. (B) Schematic map of the wg 3' UTR, showing the location of the probe used for the blot in A, and the deduced insertion site of the Sp^{revP} P-element. (C) Wing imaginal disc of an Sp^{revP}/+ individual stained with X-gal. Note that the expression pattern corresponds to that of wg.


A
Sp/+
Sp^revP/+


B
Sp^revP
wg
H B RI RV H RI B
RV
RV H B RI RV X H RI RI H X H RI
H X
probe A
1 kb


C
Sp^revP lacZ

Figure 3.3.6: Expression of *wg* in the dorsal leg disc can phenocopy the *Sp* dominant phenotype.

(A) The sternopleural region of an individual of the genotype f^{36a}; *Ubx>f+>wg/+*. Note that the f^{36a} phenotype in the macrochaetae is not completely rescued by the f^{+} cassette of the *Ubx>f+>wg* transgene, but rescue is sufficient to distinguish these bristles from bristles in which the f^{+} cassette is absent. (B) A *wg*-expressing clone occupies part of the cluster of sternopleural bristles, causing an increase in bristle number. Note the strong f^{36a} phenotype in the macrochaetae. Anterior is to the left and dorsal is up.

A
f ; Ubx>ft>wg
B
f ; Ubx>wg

When stained with X-gal, Sp^{revP} larvae produce an expression pattern that is very similar to that of the endogenous *wg* gene (Fig. 3.3.5C). This is a further indication that the P-element has inserted in the *wg* locus. In order to map the insertion site of the Sp^{revP} P-element, I probed genomic DNA from Sp^{revP} heterozygotes with DNA fragments from the *wg* 3' region, and compared the result with the original *Sp* chromosome that I used for the reversion screen (Fig. 3.3.5). This showed that the P-element has inserted in a 5 kb *Hind*III fragment located 6 kb 3' of the end of the *wg* transcript (Fig. 3.3.5). This is consistent with the observation that Sp^{revP} behaves genetically as a strong 3' regulatory mutation of *wg*, showing phenotypes similar to wg^{CX3} in combination with different *wg* alleles (Table 1).

The observation that the *Sp* dominant phenotype can be reverted by further reduction of the activity of the *wg* gene on the *Sp* chromosome suggests that *wg* is required for the generation of this phenotype. As the mutation *Sp* maps to a regulatory region of the *wg* gene and as the sternopleural bristles arise from the extreme dorsal part of the second thoracic leg disc (Schubiger, 1968), an area where *wg* is not normally expressed during development, this raised the possibility that the dominant *Sp* phenotype is due to ectopic expression of Wg. To test this possibility directly, I misexpressed *wg* during larval development in random patches of cells using the flip-out technique (Struhl and Basler, 1993), with a *wg* flip-out construct making use of the *Ubx* promoter (Diaz-Benjumea and Cohen, 1995). One adult phenotype obtained from animals treated in this way is a phenocopy of the dominant *Sp* phenotype, and in all cases this was associated with the presence of at least some *wg*-expressing cells in the cluster of sternopleural bristles (Fig. 3.3.6). This result indicates that the expression of *wg* in the vicinity of the sternopleural bristles is sufficient to generate the *Sp* dominant phenotype. *Sp* phenocopies were obtained in 20% of animals of the genotype in which clones of Wg-

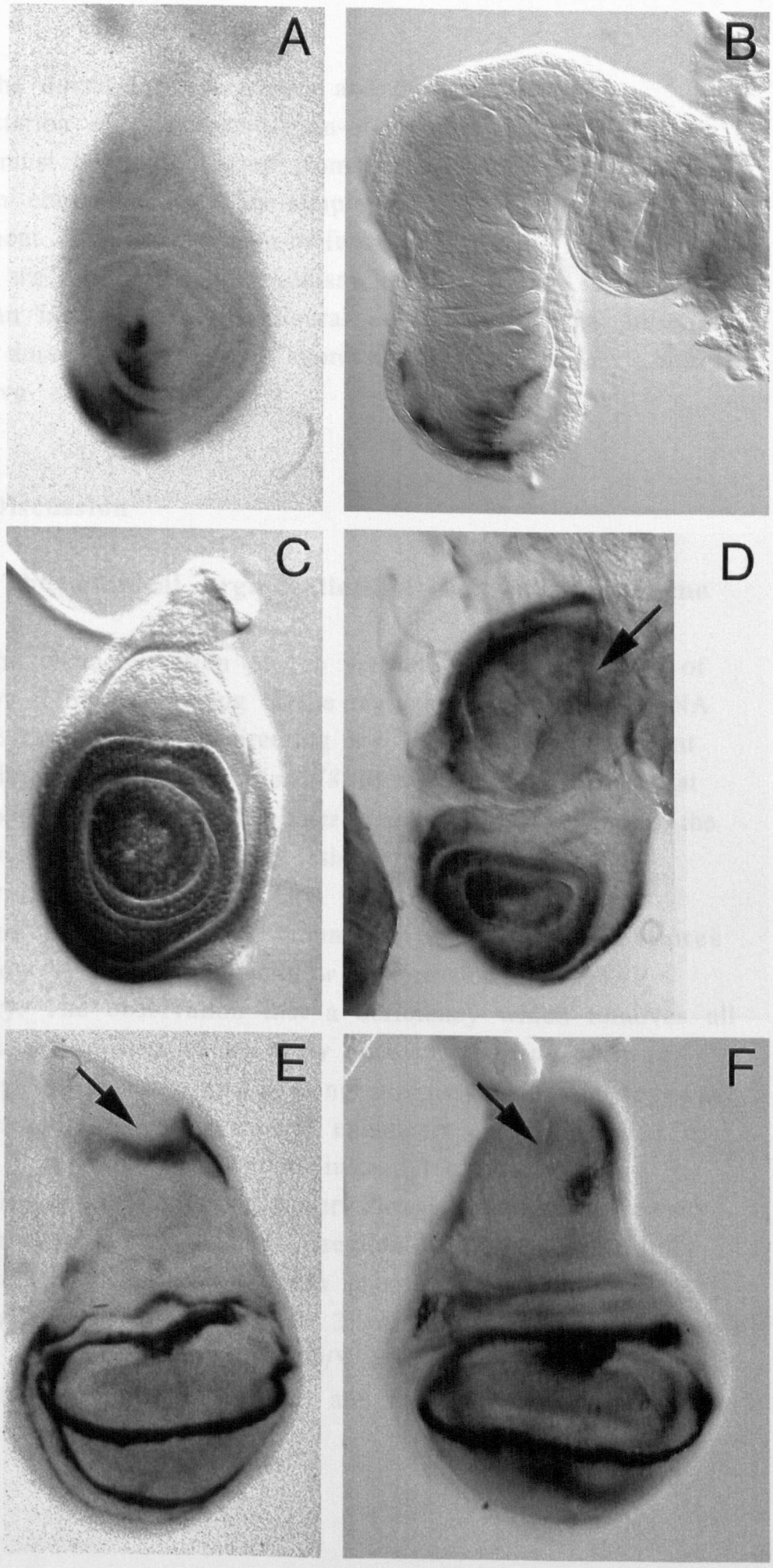
expressing cells could be generated, as compared to >50% of animals with wing phenotypes (see Diaz-Benjumea and Cohen, 1995). The difference in frequencies probably reflects the smaller number of potential target cells in the region where the sternopleural bristles are formed (as compared with the wing primordium).

To further investigate whether the *Sp* mutation causes *wg* misexpression in the dorsal second leg disc, I stained for Wg expression in both *Sp* heterozygotes and homozygotes. In *Sp* heterozygotes all leg discs showed wild-type morphology and no ectopic Wg expression could be detected (data not shown). In *Sp* homozygotes the third leg disc showed normal morphology and normal Wg expression, but the first and second leg disc pairs were clearly abnormal on the dorsal side, although no ectopic Wg expression could be detected (Fig. 3.3.7B). In some cases, the dorsal half of the disc is mildly overgrown and resembles a duplication (Fig. 3.3.7D). In other cases, this overgrowth is much more extreme (Fig. 3.3.7B). As it has been shown that expression of *wg* in the dorsal part of the leg disc can cause both bifurcations (Struhl and Basler, 1993) and overgrowth (Wilder and Perrimon, 1995), these observations support the idea that *wg* is misexpressed dorsally in the first two leg pairs of *Sp* larvae, albeit at levels that are not detectable with the most sensitive reagents available to date.

It has been shown that cells in the center of the leg disc that receive both the *wg* signal (expressed ventrally) and *decapentaplegic* signal (expressed dorsally) turn on the gene *Distal-less* (*Dll*; Diaz-Benjumea et al., 1994). I thus further characterized the *Sp* leg phenotype by looking at *Dll* expression in *Sp* homozygous first and second leg discs. In some cases, *Dll* is expressed dorsally in these discs in a manner reminiscent of bifurcations (Fig. 3.3.7D), although these rarely appeared to be distally complete. This is consistent with the suggestion that the mutation *Sp* causes ectopic *wg* activity in the dorsal first and second leg discs.

Figure 3.3.7: Expression patterns of *wg* and *Dll* in wild type and *Sp* homozygous leg and wing imaginal discs.

(A) A wild type second leg disc. *wg* RNA visualized by in situ hybridization is expressed in a ventral wedge. (B) A *Sp* homozygous second leg disc. Note the severe overgrowth on the dorsal side leading to many additional folds. *wg* RNA expression appears normally restricted to the ventral side. (C) A wild type second leg disc labeled with anti-Dll. Dll expression marks the distal segments of the leg. The folding pattern of the epithelium reflects the segments of the leg (D) A *Sp* homozygous second leg disc. Note the ectopic expression of *Dll* in an ectopic fold on the dorsal side (arrow), reminiscent of a *wg*-induced bifurcation (see Diaz-Benjumea et al., 1994). (E) A wild type wing disc labeled for *wg* RNA. The arrow indicates the stripe of *wg* expression in the notum, for comparison with the histochemical stain of the adult notum in fig. 3 (F) A *Sp* homozygous wing disc. Note the loss of *wg* expression in the notum (arrow).


A
B
C
D
E
F

The dorsal leg overgrowth and duplications are a manifestation of a recessive gain-of-function component of *Sp*, which must be distinguished from both its recessive loss-of-function component and its simple gain-of-function component. The simple gain-of-function probably leads to ectopic *wg* expression in the dorsal leg disc that is sufficient to cause an increase in sternopleural bristles, while the presence of two doses of this ectopic expression appears to cause more extensive repatterning.

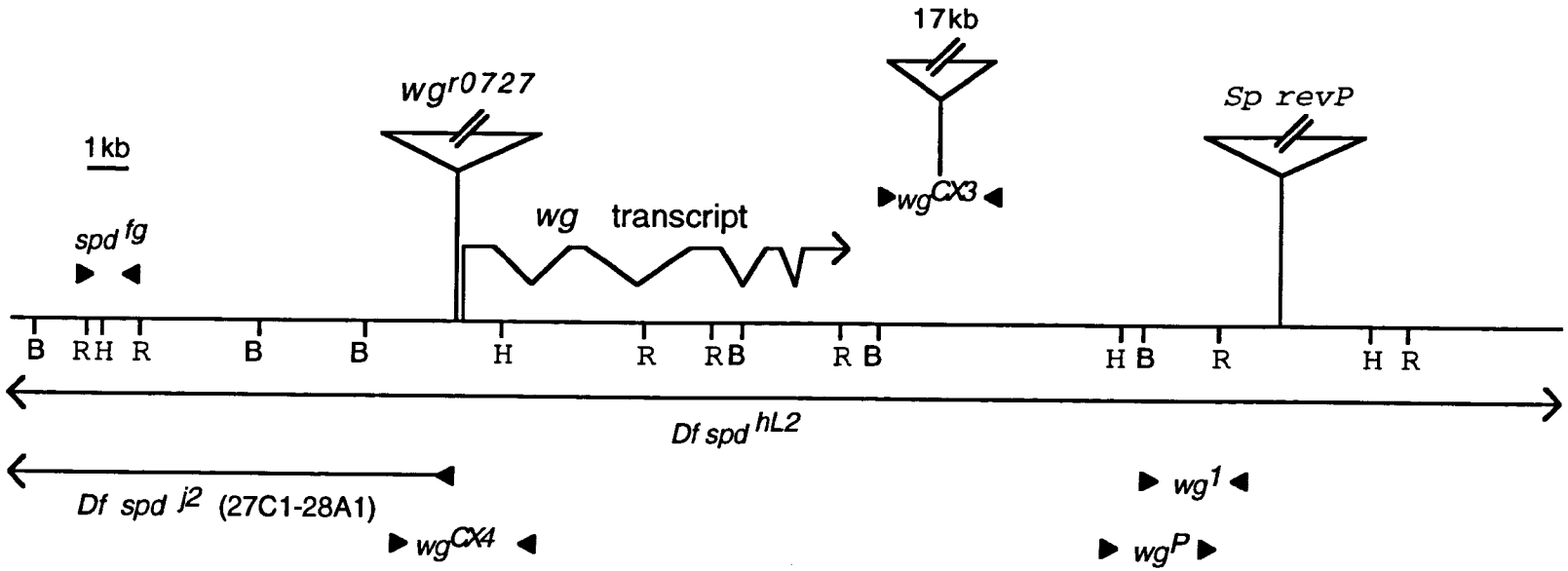
3.4: Discussion

3.4.1: The physical organization of the *wingless* gene

The characterization of the various regulatory alleles of *wg* allow a rough mapping of the regions of the flanking DNA that are responsible for directing *wg* expression in different larval domains (Fig. 3.4.1). *spdfg* identifies an enhancer that drives *wg* expression in the inner ring of the hinge and at the D/V boundary. However, expression of *wg* at the D/V boundary appears to be directed by at least two distinct enhancers that are partially redundant, as *spdfg* homozygotes only show a weak reduction of *wg* expression at the D/V boundary. The observation that a deficiency which removes all 5' regulatory regions of *wg* fully uncovers the *spdfg* hinge phenotype but not the *spdfg* wing margin phenotype suggests that the additional wing margin enhancers are not located in the *wg* 5' region. That they are instead located in the *wg* 3' region is supported by two observations. Firstly, a deficiency that removes both the 5' and 3' regions of the *wg* gene fully uncovers the *spdfg* wing margin phenotype. Secondly, the mutation *Sp*, which maps to the 3' regulatory region of *wg*, also reduces *wg* expression at the D/V boundary. These observations suggest that there are wing margin enhancers located both 5' and 3' of *wg*.

Figure 3.4.1: Physical organization of the *wg* gene.

The positions of the *wg* transcription unit and of several mutations used in this study are shown on a physical map of the *wg* locus. The locations of insertions associated with wg^{rO727}, wg^{CX3}, and Sp^{revP} are indicated by triangles. The deletions associated with *spdfg*, wg^{CX4} and wg^{1} are indicated by: > <. The proximal breakpoint of $Df(2L)spdj^{2}$ (<) is located within 100 bp of the *wg* transcription start site. $Df(2L)spdj^{2}$ and $Df(2L)spd^{hL2}$ are indicated by horizontal lines. The distal breakpoint of $In(2L)wg^{P}$ is indicated by: > >. The positions of molecular alterations in the mutants wg^{CX4}, wg^{CX3}, wg1 and wg^{P} have been described in van den Heuvel *et al.* (1993).


17kb
wg^r0727
Sp revP
1kb
wg transcript
wg^CX3
spd^fg
B RH R B B H R RB R B H B R H R
Df spd^hL2
Df spd^j2 (27C1-28A1)
wg^1
wg^CX4
wg^P

The remaining larval enhancers are likely to be located in the 3' regulatory region of *wg*. This is supported by the phenotypic analysis of the five regulatory mutations of *wg* that map to this region (wg^{CX3}, wg^{P}, wg^{1}, *Sp*, and Sp^{revP}; see Table 1 and Fig. 3.4.1). wg^{CX3} and wg^{P} reduce *wg* larval functions in the antennae, the legs, the head capsule, the notum, the abdomen, and the early function of *wg* in the wing disc (Baker, 1988; see also Table 1). These areas include nearly all of the *wg* expression domains in the larva. The two exceptions are the D/V boundary and the hinge region. Clones of cells homozygous for wg^{CX3} do not show a phenotype at the D/V boundary or in the hinge (F. Diaz-Benjumea, personal communication). This is expected, since wg^{CX3} does not affect the activity of the *spd* enhancer located 5' of *wg* that drives expression in the hinge and at the D/V boundary.

The mutation wg^{1} identifies an early function of *wg* in the wing imaginal disc (Baker, 1987, 1988a; Morata and Lawrence 1977). This early function appears to be a requirement for the specification of the wing primordium, and is associated with the expression of *wg* in an anterior ventral domain of the wing disc (Morata and Lawrence, 1977; Ng et al., 1996). Thus it is likely that the DNA deleted on the wg^{1} chromosome contains enhancer sequences driving *wg* expression in this domain. wg^{CX3}, wg^{P}, and Sp^{revP} (but not *Sp*) also uncover this function of *wg*. The mutation wg^{1} also reduces *wg* activity in the ventral head capsule (Ma and Moses, 1995).

I have presented data suggesting that *Sp* maps genetically to the 3' regulatory region of *wg*. Consistent with this suggsestion, *Sp* affects *wg* expression and activity in areas of the larva that appear to be directed by enhancers located in the *wg* 3' regulatory region: the antennae, the notum, the legs and the D/V boundary of the wing. *Sp* also reduces *wg* expression in the outer ring of the wing hinge, suggesting that this expression is also directed by enhancer(s) located 3' of *wg*.

However, no function of *wg* in this domain has been observed or described so far.

The results presented above suggest that ectopic expression of *wg* in the dorsal leg are both necessary and sufficient to cause the *Sp* dominant phenotype. Again, this is consistent with the observation that *wg* expression in the leg discs appears to be under the control of enhancer sequences located 3' of *wg*. *wg* expression in the leg is directed along the A/P boundary by *hedgehog*, and is repressed dorsally by *decapentaplegic* (Basler and Struhl, 1994; Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffman, 1996). It is thus possible that the *Sp* mutation causes expression of *wg* dorsally by attenuating the repression by *dpp*.

Although the ectopic expression of *wg* due to *Sp* is very weak, several observations suggest that cells of the leg imaginal disc are very sensitive to low levels of *wg*. Some of the methods used to date to express *wg* ectopically only supply very low levels of *wg* activity. For example, the *actin>wg* flip-out construct designed by Struhl and Basler (1993) gives levels of *wg* expression that are much lower than the endogenous *wg* expression. The UAS*wg* described by Wilder and Perrimon (1995) expresses a mutant form wg^{ts} protein, which although producing high levels of Wg antigen, appears to be compromised in its activity as compared to a UAS*wg* construct making use of a wildtype *wg* cDNA (data not shown). Both the *act>wg* and UASwg^{ts} have dramatic effects on leg development, causing bifurcations and dorsal overgrowth, but have almost no effect on wing development. However, expressing high levels of a wild-type form of *wg* in the wing disc also has a dramatic influence on these cells (Diaz-Benjumea and Cohen, 1995; see also sections 4.3 and 6.3 below). This suggests that cells of the leg disc are much more sensitive to low levels of *wg* than cells in the wing disc, and is consistent with the proposal that the low level of *wg* misexpression in *Sp* mutants is sufficient to cause the *Sp* dominant phenotype in the heterozygous state (*ie*., the

dominant *Sp* gain-of-function component), while it leads to pattern duplication and overproliferation in the homozygous state (*ie.*, the recessive *Sp* gain-of-function component).

The observation that ectopic expression of *wg* in the dorsal leg is able to induce an increase in the number of sternopleural bristles may be explained on the basis of the proposal that the *wg* pathway is able to antagonize the *Notch* pathway (Axelrod et al., 1996). These authors show that ectopic *wg* expression in the notum is able to increase the density of bristles, and that the Dishevelled protein binds to an intracellular domain of Notch. This binding may inhibit the signalling activity of Notch. Inhibition of Notch would lead to a reduction in lateral inhibition in proneural clusters, and could thus lead to an increase in the number of bristle mother cells, thereby accounting for the observed *Sp* phenotype.

3.4.2: Transvection at the *wingless* locus

As noted above, *spdfg* and *Sp* show a very complicated complementation behaviour with other *wg* alleles. For example, both *spdfg* and *Sp* are partially complemented by wg^{CX4}, a null point mutant, but not by a deficiency that removes the *wg* locus completely. Two explanations could account for these observations: 1) The *spdfg* and *Sp* mutations are not only regulatory mutations of *wg*, but simultaneously also of one or more nearby genes. Thus a *wg* null point mutant would not uncover the full phenotype of either mutation, while a deficiency including both genes would. 2) The wg^{CX4} mutant chromosome does not behave as a complete null mutant with respect to some *wg* regulatory mutants; this could be due to the intact regulatory sequences of the wg^{CX4} chromosome driving expression of the *wg* transcript on the paired sister chromosome, *ie.* by transvection. Transvection is defined as the complementation between alleles of a gene that depends on synapsis of the homologous chromosome arms (Lewis, 1954; Gelbart, 1982; reviewed in Pirrotta, 1990).

In the following paragraphs, I will discuss the available data in the light of these two possibilities.

Vis a vis the first possibility, all data suggest that the *spdfg* phenotype is only due to reduction of *wg* function. Thus it is possible to observe a reduction of *wg* expression in the phenotypically affected areas, to phenocopy the *spdfg* phenotype by reducing the activity of *wg* or of the *wg* transduction pathway, and it is also possible to rescue the phenotype by providing *wg* from another source, or by activation of the *wg* pathway. In the case of *Sp*, no rescue data are available, but loss of Wg expression is not only observed, but also sufficient to explain the *Sp* loss-of-function phenotypes. Likewise, ectopic Wg activity is both necessary and sufficient to explain the *Sp* gain-of-function phenotypes.

Vis a vis the second possibility, several observations point to the occurrence of transvection at the *wg* locus. While

spdfg is complemented by wg^{CX4}, it is not complemented by $Df(2L)spd^{J2}$, which deletes the 5' enhancer sequences of *wg* that include the *spd* enhancer. wg^{1} is another regulatory mutation of *wg* and is associated with a small deletion in the *wg* 3' regulatory region (Baker, 1988; Fig. 3.4.1). When crossed to wg^{CX4}, the *wg* null point mutant, only 15% of the resulting heterozygotes show the wg^{1} phenotype (Table 1). However, when crossed to a deficiency that uncovers *wg* completely, this percentage rises to 100% (Table 1). This is another example in which a *wg* regulatory mutation is partially complemented by wg^{CX4}.

Since transvection is pairing-dependent it is important to observe the complementation behaviour of *wg* alleles associated with chromosomal rearrangements that are expected to suppress pairing of the chromatids in the *wg* interval. wg^{P} is the only allele of *wg* that has such characteristics. It is associated with an inversion that breaks about 10 kb 3' of *wg* (vandenHeuvel et al., 1993; see Fig. 3.4.1). While wg^{CX3}, a mutation due to a 17 kb insertion in the *wg* 3' regulatory region (Fig. 3.4.1), partially complements $Df(2L)spd^{J2}$, wg^{P} does not (Table 1). This suggests that the complementation between wg^{CX3} and $Df(2L)spd^{J2}$ is pairing dependent.

However, it is important to note that formal proof of transvection would require a direct demonstration that complementation between a given pair of alleles is synapsis dependent. (*ie.*, that introducing a chromosomal rearrangement that prevents pairing also disrupts complementation).

In conclusion, the complex genetics of the *wg* locus illustrate how difficult it can be to genetically map regulatory mutations to a locus which is subject to a complex regulation involving control elements spread out over a large region of DNA, and which may, therefore, be subject to transvection. For example, a similarly complex genetic behaviour has been shown for the *dpp* gene (Gelbart, 1982). Also, the occurrence of transvection makes it necessary to reconsider the definition of

a null allele, as a null point mutant does not behave as a true null with respect to regulatory alleles, but only with respect to alleles that compromise the transcript itself, or in the absence of pairing of the chromatids.

CHAPTER 4:
WINGLESS FUNCTIONS AS A LOCALIZED MITOGEN IN THE DEVELOPING WING HINGE

CHAPTER 4: WINGLESS FUNCTIONS AS A LOCALIZED MITOGEN IN THE DEVELOPING WING HINGE

4.1: Introduction

An important question in developmental biology is how positional information is used to control cell proliferation. How is cell proliferation integrated with patterning events, how are final cell numbers specified, and how are proportions regulated? As discussed in section 1.3, the basic principles by which pattern is generated in the appendages of Drosophila are starting to be understood, making this developmental process a good starting point for examining the coordination of proliferation with patterning. In this chapter, I present data suggesting that Wingless may function as a localized mitogen in the developing wing hinge, thus translating positional information into the local control of cell proliferation..

Wingless may have an exclusively mitogenic function in the hinge, which contrasts with its fate-specifying function in many other contexts. These differences are similar to region-specific differences in mitogenic and fate-specification responses to Wnt-1 in the vertebrate CNS (Dickinson et al., 1994), highlighting the importance of understanding how different cells respond differently to the same signal, and thereby achieve specificity in the regulation of development.

4.2: *wingless* is required for normal growth of the wing hinge

As described in the previous chapter, *spdfg* is a regulatory mutation of *wg* that strongly reduces *wg* expression in the inner ring of the wing hinge, as a consequence of which specific structures of the distal hinge in the adult wing are lost. Not only the structures in the distal hinge are lost, but also the cells that would normally be found in this area. The easiest way to describe this phenotype is to imagine a wild-type hinge in which the distal hinge has been cut out, followed by the splicing together of the cut edges, resulting in a decrease in the overall size of the hinge (see Fig.3.2.1). This suggests that the absence of *wg* activity in the inner ring results in the loss of specific cells, and not only of specific fates. There could be at least two possible explanations for this effect. Either the absence of *wg* leads to elevated cell death in this region of the disc, thus causing the loss of the cells after they have been generated. Alternatively, the cells are never generated in the first place. To distinguish between these possibilities, I further characterized the *spdfg* phenotype.

The expression of the enhancer trap *A101* (inserted in the gene *neuralized*, see Ghysen and O'Kane, 1989; Huang et al., 1991) marks cells of a neuronal fate in the imaginal discs. In late third instar wing discs of *spdfg* mutants, specific groups of sense-organ precursors, including all of part of the Sc12 group of campaniform sensillae, are missing (Fig. 4.2.1). These cells are inside, or close to the inner ring of *wg* expression in wild-type wing discs. Examination of the *spdfg* phenotype also shows that there is tissue missing in this area, bringing the Sc25 group of campaniform sensillae closer to the sensillae located on vein 3 (compare Fig. 4.2.1B with 4.2.1D). This indicates that the structures deleted in the adult hinge are already absent in late third instar wing imaginal discs of *spdfg* mutants.

Figure 4.2.1: The hinge phenotype in *spdfg* mutants does not appear to be due to cell death.

A-F were stained with X-gal. (A) *A101* expression in a wild type late third instar wing imaginal disc. (B) A close up view of the dorsal hinge region of the disc shown in A. The SMCs of the Sc25 group of campaniform sensillae are labeled, and the SMCs of the sensillum of the anterior cross vein (ACV) and the second sensillum of the third vein (L3-2) are marked with asterisks. The SMCs which lie between these two groups correspond to the giant sensillum of the dorsal radius (GSR) and the Sc12 group of campaniform sensillae. Assignment of SMCs is according to Huang et al. (1991). The third instar wing imaginal disc has several characteristic folds, a pair of which are marked by arrows here and in panels D and F. (C) *A101* expression in a *spdfg* late third instar wing imaginal disc. (D) Close up view of the dorsal hinge region of the disc shown in C. Note that tissue between the two folds marked by arrows appears to be missing in *spdfg*, bringing the Sc25 SMCs closer to the ACV and L3-2 (asterisks). The GSR is absent, while the Sc12 group are partially absent. In this genetic background there is an enhancement of the *spdfg* phenotype that results in scalloping of the anterior and posterior wing margins. For this reason, SMCs of the proximal anterior wing margin are missing in the mutant. (E) Expression of *lacZ* driven by the *spd* enhancer in a *spdfg* mutant wing disc. Note that the expression is identical to that in a wild-type background (compare with 5D). (F) A close up view of the dorsal hinge region of the disc shown in E. The inner ring of *wg* expression lies roughly midway between the two folds that are marked by arrows in B,D and F. Note that even though the reduction in tissue between the two folds is clearly visible in the mutant, the *spd* enhancer still drives strong expression here, suggesting that loss of the folds cannot be due to cell death.

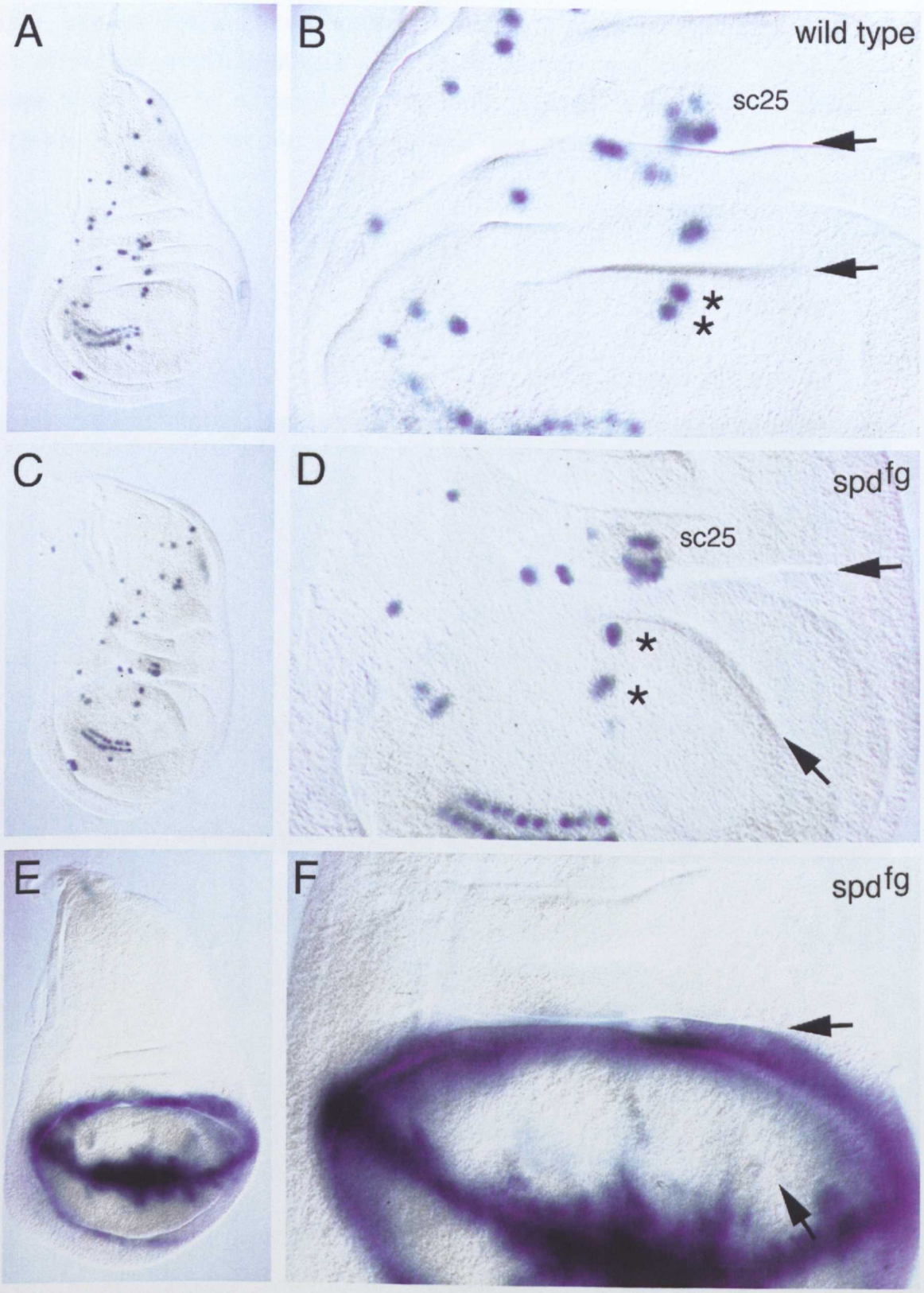
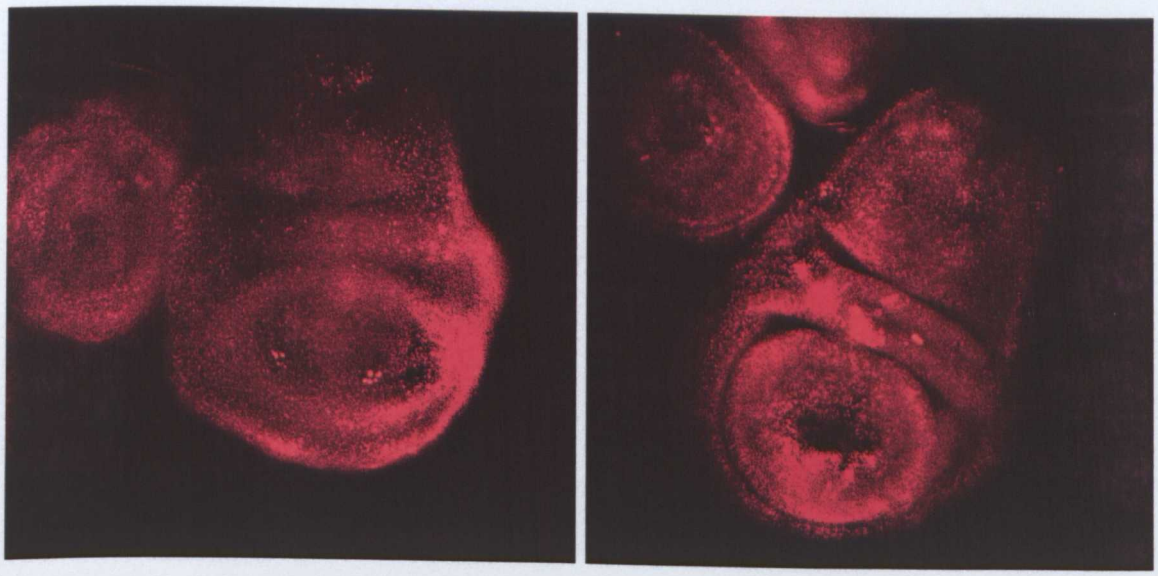

A
B
wild type
sc25
C
D
spd^fg
sc25
E
F
spd^fg

Figure 4.2.2: Acridine orange staining of *spdfg* mutant discs suggests that the *spdfg* phenotype is not due to elevated cell death.

Two different examples of *spdfg* mutant wing discs stained with acridine orange are shown. Note that although there are cells that label with acridine orange, they do not localize to the hinge area, and the levels of cell death are not elevated above wild-type levels (data not shown).

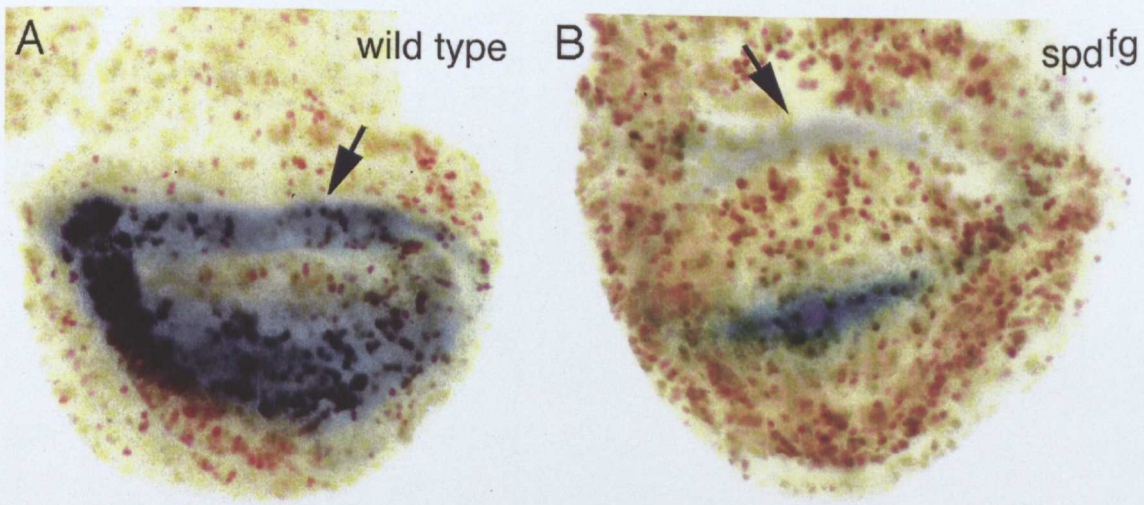


To determine whether the loss of distal hinge structures in *spdfg* is due to cell death, I stained imaginal discs of *spdfg* mutants with acridine orange, which labels apoptotic nuclei (Masucci et al., 1990). No abnormal cell death was observable in any part of the disc throughout third instar (Fig. 4.2.2). About fifty wing discs from larvae spread over third instar stages were examined. This suggests that the phenotype is not due to cell death, or that the increase in the numbers of dying cells is too subtle or too rapid to be detected. To further address this question, I examined the expression of the *spd* enhancer-*lacZ* reporter gene in a *spdfg* mutant background. The *spd* enhancer-*lacZ* reporter gene is expressed in the center of the stripe of tissue that is deleted in *spdfg* mutants. Hence, if the absence of *wg* activity in the inner ring leads to localized cell death, it is expected that, after correct initial activation, the expression of this reporter would fade away, as the cells in which it is expressed would die. However, this was not the observed result. During late third instar, when the loss of tissue in the hinge of *spdfg* individuals is already apparent, the enhancer is still strongly expressed (Fig. 4.2.1F). It continues to be strongly expressed in the pupal and adult wings of mutants (data not shown).

These data suggest that the *spdfg* hinge phenotype may not be due to cell death and raise the possibility that it is instead due to reduced local proliferation. To examine this possibility, I double-labelled *spdfg* mutant discs for the expression of the *spd* enhancer-*lacZ* reporter, as well as for BrdU incorporation. The *spd* enhancer-*lacZ* marks the cells of interest, while BrdU marks dividing cells. In wild-type discs, many cells expressing the *lacZ* reporter can be seen to divide (Fig. 4.2.3A). In the *spdfg* mutant background, however, only very few cells expressing the *lacZ* repoter gene are dividing (Fig. 4.2.3B).

Figure 4.2.3: Underproliferation in the hinge of *spdfg* mutants revealed by BrdU labelling

A and B were double labelled with X-gal and BrdU. (A) BrdU pulse-labeling of a wild type mid-third instar disc showing the pattern of cell division in relation to the domain of expression of the *spd* enhancer. Note the presence of labelled nuclei (brown) in the cells expressing the enhancer (blue). (B) BrdU pulse-labeling of a mid-third instar *spd* mutant disc. Note the relative absence of cell division in the dorsal hinge region (arrow). Enhancer driven lacZ expression was understained to emphasize the absence of incorporation of BrdU.


A
wild type
B
spd fg

Taken together, these results suggest that *wg* activity in the inner ring is required to promote local cell proliferation in the wing hinge.

Consistent with this proposal, *armadillo* clones die in the wing pouch, but not in the hinge region (Fig. 6.3.2). *armadillo* (*arm*) is required to transduce the *wg* signal, and *arm* clones die in the wing pouch because they lose *vestigial* expression (Fig.6.3.1). Clones in the hinge region can be recovered, but they are generally small, suggesting that activity of the *wg* pathway is not required for survival, but for proliferation of the cells of the hinge.

4.3: Ectopic activation of the *wingless* pathway in the hinge region induces overproliferation

It has been shown that activation of the *wg* pathway is able to induce wing margin fates in the wing pouch (Simpson et al., 1988; Blair, 1992; Phillips and Whittle, 1993; Couso et al., 1994; Diaz-Benjumea and Cohen, 1995). To determine if *wg* is also sufficient to specify pattern elements in the hinge region, we misexpressed *wg* using the GAL4:UAS system (Brand and Perrimon, 1993). As a GAL4 driver we used MS1096, which has been shown to express GAL4 in the dorsal wing pouch (Capdevila and Guerrero, 1994). However, we found that MS1096 is also expressed in the ventral wing pouch, as well as in the dorsal hinge, although at lower levels (Fig. 4.3.1A). When crossed to this driver, UAS*wg* is able to activate *Distal-less* (*Dll*) expression throughout the wing pouch (Fig. 4.3.1C). *Dll* has been shown to be a target of *wg* in the wing pouch (Diaz-Benjumea and Cohen, 1995). As expected, pharate adults recovered from this cross also have a high density of wing margin bristles on both wing surfaces, although sometimes with a lower density on the ventral side (Fig. 4.3.1D). I also observed that there is an increase in the size of the dorsal hinge in this combination (visualized by the area that

expresses UAS*lacZ* and UAS*wg* under the control of MS1096 in the dorsal hinge, but does not express Dll, Fig. 4.3.1C). This correlates with a broadening of the hinge region of the pharates (compare Fig. 4.3.1B with 4.3.1D), suggesting that there may be extra growth in the hinge induced by ectopic *wg* activity.

dishevelled (*dsh*) is required to transduce the *wg* signal (see Introduction and Fig. 3.2.5). Dsh is a phosphoprotein that becomes hyperphosphorylated in response to the *wg* signal, and it has been shown that overexpression of Dsh is sufficient to cause its hyperphosphorylation and activation of the *wg* pathway, as assayed by the accumulation of Armadillo protein in cultured cells (Yanagawa et al., 1995). Sokol et al. (1995) have shown that injection of *Xdsh* mRNA into Xenopus oocytes mimics the effect of injection of *Wnt* mRNA, even though *Xdsh* mRNA is present ubiquitously in the oocyte. These results suggested that overexpression of *dsh* in *Drosophila* could have a similar effect. Indeed, expression of UAS*dsh* by several GAL4 drivers can phenocopy ectopic expression of *wg* in all imaginal tissues (Figs. 4.3.1, 6.3.3, and data not shown; see also Axelrod et al.,1996). Park et al. (1996) have shown that overexpression of Dsh in the Drosophila embryo can trigger the *wg* pathway in a *wg* null mutant background (*ie.* in the absence of Wg protein).

UAS*dsh* crossed to MS1096 induces Dll expression throughout the wing pouch, as observed with UAS*wg* (Fig. 4.3.1E; see also dppGAL4:UASdsh, Fig. 6.3.3B and C). The dorsal hinge is greatly overgrown in this combination, as indicated by the abnormal folds of cells expressing UAS*lacZ* and UAS*dsh*, but not Dll (Fig. 4.3.1E). While most individuals of this phenotype die at larval or early pupal stages, a few survive to late pupal (pharate) stages. These individuals have densely packed wing margin bristles all over the wing blade surfaces, although in some cases the ventral surface is more sparsely covered (Fig. 4.3.1F). The region occupied by the proximal

dorsal hinge is greatly expanded. This effect of UAS*dsh* is much stronger than that of UAS*wg*.

To derermine whether the expansion of the dorsal hinge in MS1096:UAS*dsh* individuals is due to increased proliferation, I pulse labelled imaginal discs with BrdU. When incubated with BrdU for 10 minutes, only few cells are labeled in a wildtype wing disc (not shown). By contrast, both the frequency and the intensity of labeling is increased in the dorsal hinge of MS1096:UAS*dsh* wing discs (compare the region between the arrows in Fig. 4.3.2A with the regions on either side, which resemble control discs). This result indicates that overexpression of *dsh* in the hinge region of the wing disc strongly stimulates cell division.

Examination of the extra tissue in the proximal hinge of MS1096:UAS*dsh* pharates indicates that it is not patterned. There does not appear to be a duplication of distal hinge structures more proximally. To further address this point, I crossed the neuronal marker *A101* (see Fig. 4.2.1 above) into this background (Fig. 4.3.2B). It is possible to locate most of the sensillar precursors in the hinge region in the correct location in these discs and in the correct numbers. However, the spacing of cells within clusters of sensillae is abnormal. Thus the Sc25 group of sensillae appears as a compact cluster in wild-type discs, but is stretched out into a long line in discs of the genotype MS1096:UAS*dsh* (compare Fig. 4.2.1A with 4.3.2B). These results suggest that ectopic activation of the *wg* pathway in the hinge region of the wing does not respecify pattern, but instead stimulates cell division. However, the greatly increased cell numbers disrupt the organization of the hinge.

Figure 4.3.1: Over-growth of the wing hinge caused by ectopic expression of *wg* or *dsh*.

A was stained with X-gal, while C and E were stained with anti Dll antibody and X-gal. (A) Expression of the Gal4 driver MS1096 in the wing visualized by UAS-*lacZ*. Note the strong blue label in the dorsal wing pouch, and the weaker (pink-blue) labeling in the ventral wing and dorsal wing hinge regions.
(B) Cuticle from a wild-type phatate adult. t indicates the tegula.
(C) MS1096:UAS-*wg*+UAS-*lacZ* wing disc double labeled for *lacZ* activity (blue) and Dll protein expression (brown). In wild type discs Dll is expressed in a narrow band of cells along the wing margin under control of *wg* (Diaz-Benjumea & Cohen 1995). Note that Dll is expressed throughout the entire wing pouch, but not in the overgrown wing hinge region. Thus cells in the hinge respond differently to the Wg signal than cells in the wing blade. The lower level of *wg* expression in the ventral wing is still sufficient to respecify cell fates to direct Dll expression. The overlap of Dll and *lacZ* staining is more difficult to see in the dorsal wing pouch due to the higher level of *lacZ* activity.
(D) Cuticle from a MS1096:UAS-*wg* pharate adult. Cells throughout the wing pouch differentiate structures characteristic of the wing margin. Note the dense cluster of anterior wing margin bristles (white arrow). The tegula shows an increase in bristle density (black arrow). The proximal wing hinge is expanded (compare with B), but does not show any pattern duplication.
(E) MS1096:UAS-*dsh*+UAS-*lacZ* wing disc double labeled for *lacZ* activity and Dll protein expression. Note the vast expansion of the dorsal wing hinge, visible as a set of unusual folds in the disc epithelium. The cells contributing to the overgrowth express *lacZ* indicating that they come from the region where MS1096 is expressed. Dll is expressed throughout the wing pouch.
(F) Cuticle from a MS1096:UAS-*dsh* pharate adult. Note the vast over-growth of the proximal wing hinge (asterisk). This consists of apparently unpatterned tissue.

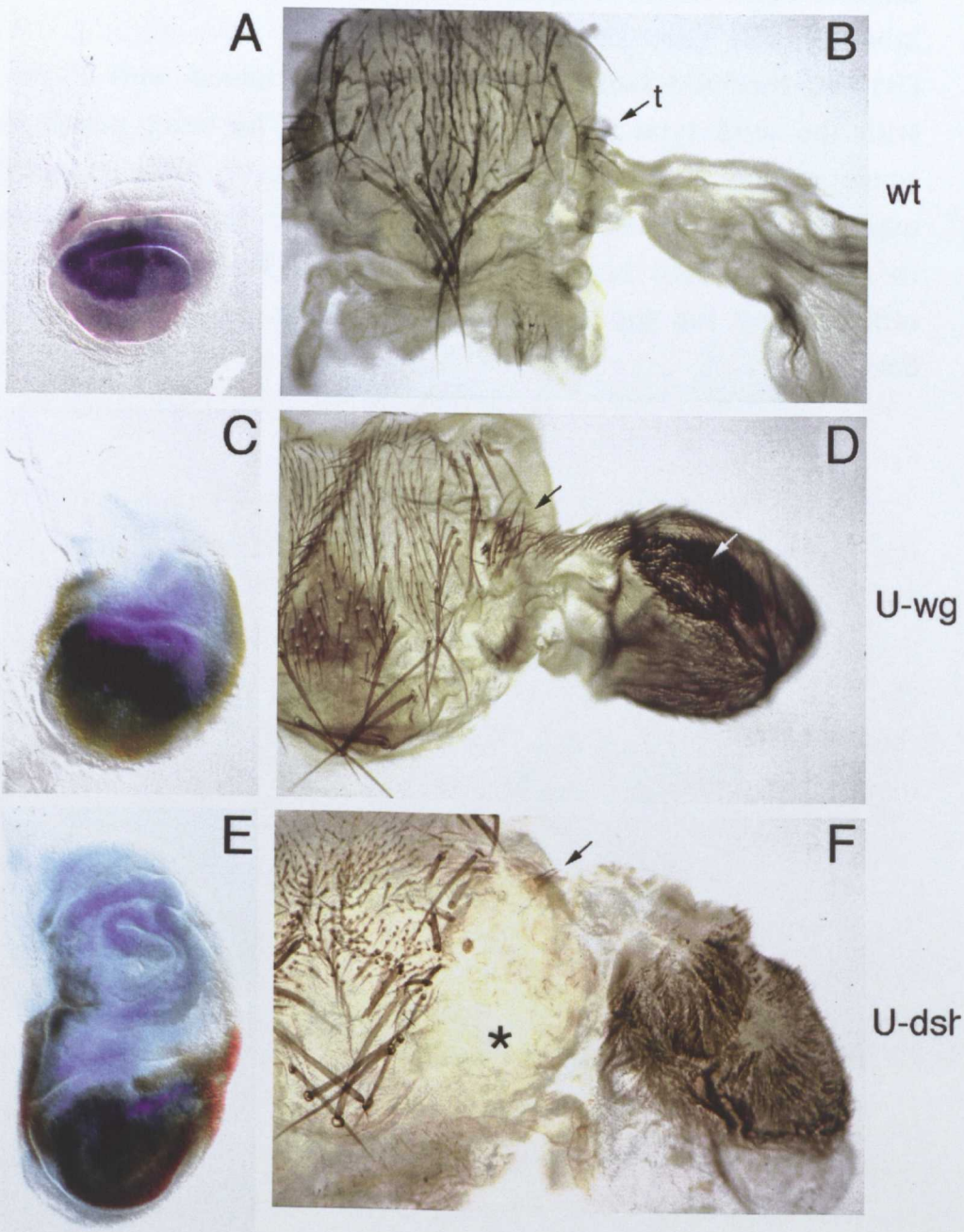
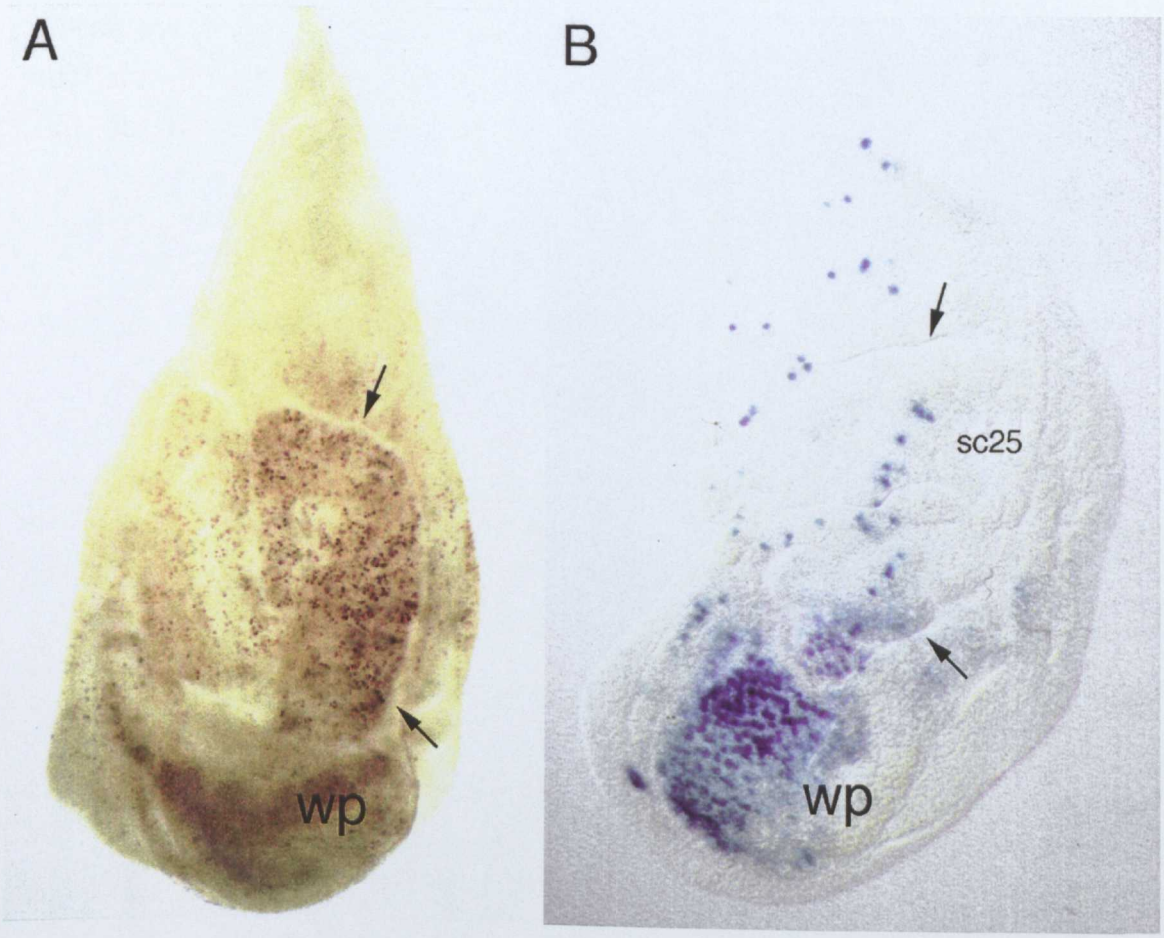

A
B
t
wt
C
D
U-wg
E
F
*
U-dsh

Figure 4.3.2: Over-proliferation of the wing hinge caused by UAS-*dsh*

(A) MS1096:UAS-*dsh* wing disc pulse labeled by BrdU incorporation shows an elevated level of cell division in the wing hinge region (arrows). The wing pouch (wp) and notum show a lower density of labeled cells. Control discs do not show the increased density of labeled cells (not shown).
(B) MS1096:UAS-*dsh*; A101 wing disc stained with X-gal (compare with the wild type pattern in fig 6A,B). The wing pouch is filled with sense organs, indicating that cell fates have been respecified toward margin. By contrast, the pattern of sense organ precursors is normal in the overgrown hinge region (arrows). For comparison, the fold indicated by the top arrow is the same as the fold at the top of panel 6B.


A
wp
B
sc25
wp

4.4: Discussion

4.4.1: Distinct mitogenic and cell fate specification responses to the Wingless signal in different regions of the wing disc

The data presented in this chapter suggest that *wg* function is both necessary and sufficient to promote proliferation of cells in the wing hinge. Reduction of *wg* activity (or of the *wg* pathway) leads to underproliferation in a region of the wing imaginal disc that will give rise the distal hinge of the adult wing. Conversely, ectopic expression of *wg* (or activation of the *wg* pathway) leads to local overgrowth of the region of the wing disc that gives rise to the proximal hinge. As no respecification of pattern elements of the proximal hinge can be detected under these conditions, it may be that *wg* exclusively has a mitogenic effect on these cells. Thus the loss of specific pattern elements in the distal hinge of *spdfg* mutants could be an entirely indirect effect, due to the fact that the cells which are the "raw material" for the patterning of this region are absent because of underproliferation. Alternatively, *wg* could not only have a mitogenic function in the hinge, but also play a permissive role in pattern specification of specific hinge structures. This would be similar to the permissive effect that wg has in the specification of several bristle precursors in the notum (Phillips and Whittle, 1993; see also Introduction), but would be different from the inductive effect of *wg* in the wing pouch, where it is sufficient to induce wing margin bristle cell fates (see Introduction).

On the basis of the cellular competence to respond to the *wg* signal, the wing imaginal disc can be subdivided into at least three distinct populations of cells: the notum (or thorax), the hinge, and the wing pouch (or wing blade). Cells in each of these regions respond differently to *wg*. As already noted above, *wg* is required but not sufficient for the specification of

several macrobristles in the notum. Neither the removal nor the ectopic expression of *wg* has any effect on proliferation in the notum (Couso et al., 1993; Phillips and Whittle, 1993; Ng et al., 1996; data not shown). In the wing pouch, *wg* induces wing margin cell fates as well as proliferation, and I will discuss evidence that it elicits different responses in a concentration-dependent manner in chapter 6 below (see also Zecca et al., 1996; Neumann and Cohen, 1997). The effect of *wg* in the hinge is different yet again. It induces proliferation but apparently not cell fates. Strong ectopic expression of *wg* throughout the dorsal wing and hinge reveals that there appears to be a straight line of demarcation between the hinge and the wing pouch: cells on the wing pouch side of this line express *Distal-less*, *vestigial* and the *acheate-scute* complex genes in response to *wg*, while cells on the other side do not (Figs.4.3.2B and 6.2.2).

These observations raise the question of how the differences in competence are controlled. It may be that different 'selector genes' similar to *engrailed* or *apterous* are expressed in each of these regions. Indeed the boundary between the notum and the hinge appears to be a lineage boundary similar to those that divide the disc into A/P and D/V compartments (Garcia-Bellido et al., 1973). However, there is no good evidence for such a compartment boundary between cells of the hinge and the wing blade.

The hinge itself can be further divided into at least two populations of cells. All cells in the hinge appear to have a similar competence to respond to *wg* by undergoing division, but while cells in the distal hinge depend on *wg* for their normal proliferation, cells in the proximal hinge do not. This suggests that other factor(s) than *wg* regulate proliferation in the proximal hinge. The grotesque overproliferation induced in response to activation of the *wg* pathway in the proximal hinge may thus be a product of synergy between *wg* and this other factor. Also, overexpression of *dsh* is much more potent than overexpression of *wg* in inducing overproliferation in the

hinge. This could be explained if there is a component in the *wg* pathway that is expressed at a low level in the hinge and that is downstream of *wg* but upstream of *dsh*. This could be the *wg* receptor or even *dsh* itself.

An interesting observation is that the regulation of proliferation in the distal hinge is under the control of locally expressed *wg*, and is independent from the regulation of proliferation in the rest of the disc. This is different from the proliferation in the wing pouch, which appears to be regulated at a distance by long-range signalling by *dpp* and *wg*, secreted from the A/P and D/V boundaries, respectively.

Wnt-1 was originally identified as a proto-oncogene, able to induce overproliferation in mouse mammary epithelial cells (reviewed in Nusse and Varmus, 1992). It was subsequently shown that Drosophila Wg has an identical mitogenic effect on mouse mammary epithelial cells (Ramakrishna and Brown, 1993). Also, Skaer and Martinez-Arias (1992) have shown that *wg* is required for the normal proliferation of cells in the anlage of the Drosophila Malphigian tubules. The observation that only a subset of the cells in the wing disc are able to respond to *wg* by dividing is interesting because similar differences in competence have been observed in vertebrate cells. Thus *Wnt-1* stimulates proliferation in some vertebrate cell lines, but not in others (Jue et al., 1992). Also, region-specific differences in mitogenic and cell fate specification responses to *Wnt-1* have been observed in the mouse CNS (Dickinson et al., 1994). *Wnt-1* is expressed at the midbrain-hindbrain boundary and is required in this region for the regulation of *engrailed* expression and cell fate specification. *Wnt-1* is also expressed in the dorsal-most cells of the neural tube, where it may have a redundant function, as no phenotype is observed in this region in *Wnt-1* kmutant mice. However, when *Wnt-1* is ectopically expressed in the ventral neural tube using a HOXb-4 Region A enhancer, this causes overproliferation without cell fate respecification (as assayed by the expression of markers that are expressed differentially

along the D/V axis of the neural tube). This effect of *Wnt-1* is very similar to the effect of *wg* in the wing hinge. Since *Wnt-1* and *wg* have practically identical effects in all assays available so far, it is likely that the pathways by which cells distinguish between growth and cell fate specification in response to *Wnt* signals is conserved between vertebrates and invertebrates. It will be interesting to identify the factors that control these choices.

CHAPTER 5:
THE POSITION OF WINGLESS IN THE GENETIC HIERARCHY THAT ESTABLISHES THE D/V ORGANIZER

CHAPTER 5: THE POSITION OF WINGLESS IN THE GENETIC HIERARCHY THAT ESTABLISHES THE D/V ORGANIZER

5.1: Introduction

A large body of data suggests that interactions between dorsal and ventral cells in the Drosophila wing imaginal disc establishes an organizing center that directs pattern formation and growth of the wing pouch (reviewed in Blair, 1995; Brook et al., 1996). This process is mediated by a complex genetic hierarchy, which I will attempt to review briefly here.

Activity of the transmembrane receptor *Notch* is critical for wing development (Schellenbarger and Mohler, 1978) and is required in cells abutting the D/V boundary (De Celis and Garcia Bellido, 1994). Several published observations have suggested that *Notch* functions to establish *wg*, *vestigial* (a novel nuclear protein) and *cut* (a homeodomain transcription factor) expression at the D/V boundary. *Serrate*, a ligand of *Notch* (Rebay et al., 1991), is expressed in cells of the dorsal compartment and activates expression of *wg*, *vestigial* and *cut* in nearby ventral cells (Kim et al., 1995; Diaz-Benjumea and Cohen, 1995; Couso et al., 1995; De Celis et al., 1996a; Doherty et al., 1996). Also, clones of cells that lack *Notch* activity lose *wg* expression autonomously at the D/V boundary (Rulifson and Blair, 1995). *Delta*, another ligand of *Notch* (Fehon et al., 1990; Rebay et al., 1991), is required for the activation of *wg*, *vestigial* and *cut* in dorsal cells abutting the boundary (De Celis et al., 1996a; Doherty et al., 1996).

These results strongly suggest that the local activation of the *Notch* receptor in cells abutting the D/V boundary establishes the D/V organizer through the induction of the genes *wg*, *vestigial* and *cut*. However, data have been presented that *wg* is sufficient to mediate all the organizing activities of the D/V organizer (Diaz-Benjumea and Cohen, 1995), and that the gene *cut* is downstream of *wg* (Couso et al., 1994).

I wished to clarify the regulatory relationships between *Notch*, *wingless*, *vestigial* and *cut*. In this chapter, I present data suggesting that *wingless* and *vestigial* are idependent targets of Notch at the D/V boundary. Subsequently, *vestigial* is expressed in a broad domain that fills the wing pouch. This second phase of *vestigial* expression depends on *wingless* function at the D/V boundary. In addition, Notch and Wingless cooperate to activate the expression of *cut* at the D/V boundary. These data allow a more accurate definition of the position of Wingless in the genetic hierarchy that organizes the D/V axis of the wing.

5.2 Activity of the Notch signalling pathway is both necessary and sufficient for the activation of *wingless* and *vestigial* expression at the D/V boundary

Fig. 5.2.1 summarizes the effects of the activity of the *Notch* pathway on the expression of *wg*, *vestigial* and *cut*. *Suppressor of Hairless* encodes a DNA-binding protein that is thought to transduce the *Notch* signal (Fortini & Artavanis-Tsakonas, 1994; Lecourtois and Schweisguth, 1995; Bailey & Posakony, 1995). *Suppressor of Hairless* mutant wing imaginal discs fail to activate *wg*, *cut* and an enhancer of *vestigial* that is expressed at the D/V boundary in the wing pouch (Fig. 5.2.1B,F,J). Couso et al. (1995) have also shown that no Vestigial protein is expressed in the wing pouch of *Suppressor of Hairless* wing discs. Conversely, if a ligand-independent activated form of *Notch* (Struhl et al., 1993), UAS*Notch(intra)*, is expressed under the control of the GAL4 driver MS1096 (which is expressed most strongly in the dorsal wing pouch, see Fig. 4.3.1A), *wg*, *cut* and the early *vestigial* enhancer are ectopically activated.

Figure 5.2.1: Notch-dependent expression of *wingless, cut* and the early *vestigial* enhancer.

(A,E,I) wild-type wing discs. (B,F, J) *Su(H)*^{AR9}*/Su(H)*^{SF8} mutant wing discs. (C, G, K) wing discs expressing UAS*Notch(intra)* under control of MS1096. (D,H) MS1096:UAS*wg*$^{+}$ wing discs. All discs are mature third instar. (A-D) *cut-lacZ* expression. (E-H) *vestigial* early enhancer-lacZ expression. To distinguish the initial activation of *vestigial* expression at the D/V boundary from subsequent expression in the wing blade we have made use of a boundary-specific early enhancer directing lacZ expression (vg-int2 lacZ; Williams et al. 1994). (I-K) *wg* enhancer-lacZ expression. A specific enhancer from the *wg* gene was used to direct lacZ expression in the wing hinge and at the D/V boundary (spd-lacZ). All three reporter genes are lost form the wing margin in *Su(H)* mutant discs (B,F, J), and are misexpressed in the dorsal compartment when the active form of Notch is misexpressed under MS1096 control (C, G, K). Neither *cut* or *vestigial* are misexpressed in MS1096:UAS*wg* discs (D,H).

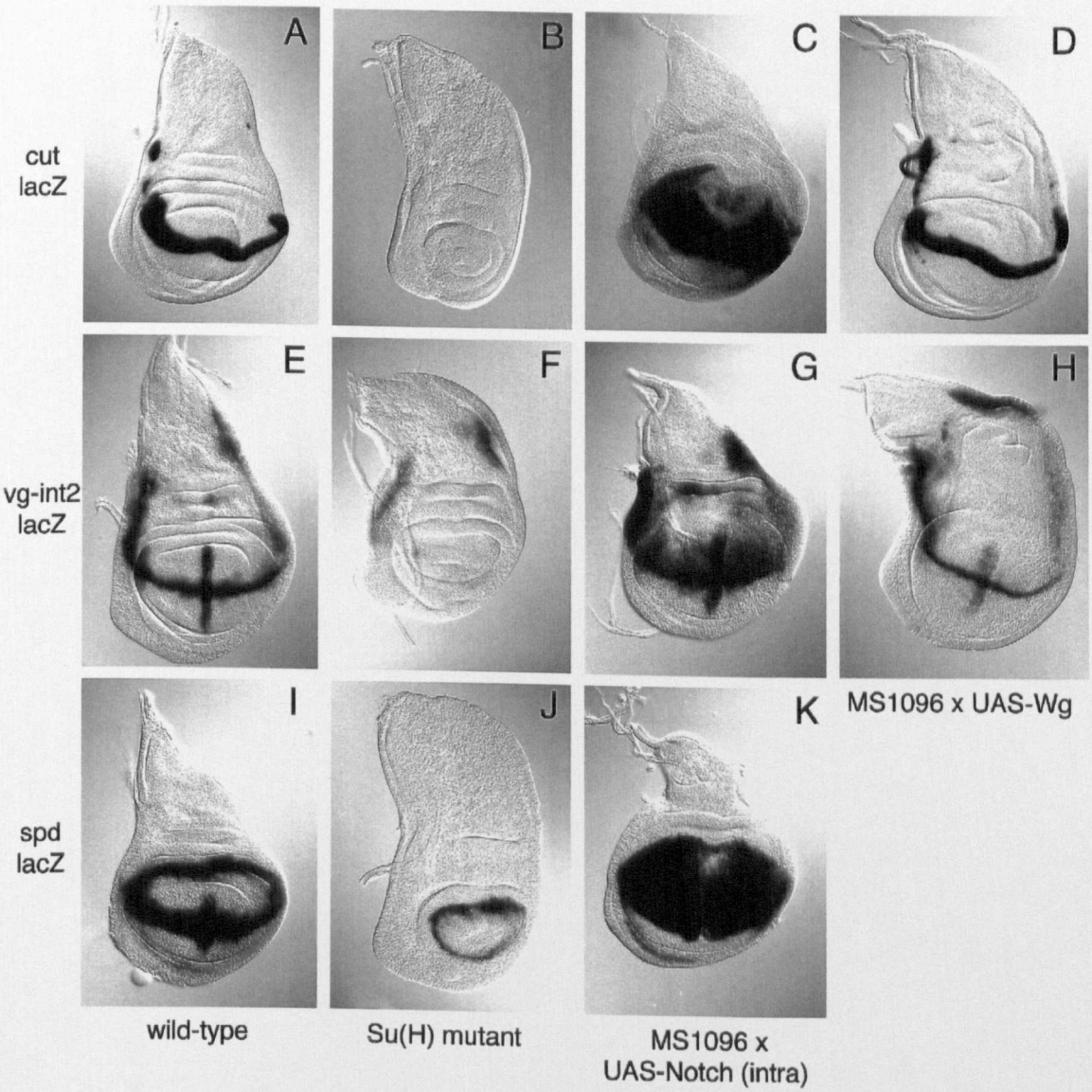

A
B
C
D
cut
lacZ
E
F
G
H
vg-int2
lacZ
MS1096 x UAS-Wg
I
J
K
spd
lacZ
wild-type
Su(H) mutant
MS1096 x
UAS-Notch (intra)

Figure 5.2.2: Cell-autonomous loss of *vestigial, wingless* and *cut* expression in *Su(H)* mutant clones

(A) *vestigial-lacZ* expression (red). *Su(H)* mutant cells are visualized by the absence of N-myc expression (green in top panel, shown separately below). Bright green cells are twin spots carrying two copies of the N-myc transgene).
(B) *wg-lacZ* expressing cells (red). *Su(H)* mutant cells are visualized by the absence of N-myc expression (green).
(C,D) *cut-lacZ* expressing cells (red). Note the absence of *cut* expression even in very small clones of *su(H)* mutant cells (arrows in D). Both clones in D are ventral only. The clone on the left has autonomously lost *cut* expression, while the clone on the right has both autonomously lost *cut* expression and caused non-autonomous loss of *cut* expression in wild-type cells on the dorsal side of the D/V boundary (as described for *Notch* mutant clones with respect to *wg* expression, Rulifson and Blair, 1995). Lower panel, N-myc single channel image.

Vg-lacZ
Wg-lacZ
cut-lacZ
A
B
C
D

Clones of *Suppressor of Hairless* mutant cells cause loss of wing tissue and scalloping of the wing, but only if cells at the D/V boundary are mutant (Diaz-Benjumea and Cohen, 1995; De Celis et al., 1996a), as previously described for *Notch* mutant clones (De Celis and Garcia Bellido, 1994). Consistent with this observation, *Suppressor of Hairless* mutant clones autonomously lose expression of *wg-lacZ*, *cut-lacZ*, and of the *vestigial* D/V boundary enhancer (Fig. 5.2.2). These results indicate that *wg*, *vestigial* and *cut* are targets for activation by a *Notch*-dependent signal, transduced through *Suppressor of Hairless*.

5.3: *wingless* and *vestigial* are parallel targets of the Notch pathway at the D/V boundary

wg and the early *vestigial* enhancer are expressed at the D/V boundary beginning in second instar (Couso et al., 1993, 1995; Williams et al., 1993, 1994; Ng et al., 1996). I wished to determine whether *wg* and the early *vestigial* enhancer are independent targets of the *Notch* pathway, or if they can be placed in a regulatory hierarchy. To ask if *wg* regulates the early *vestigial* enhancer, I misexpressed *wg* using the GAL4:UAS system (see Brand and Perrimon, 1993). *wg* activity is required for the formation of wing margin bristles (Phillips and Whittle, 1993; Couso et al., 1994) and ectopic activation of the *wg* pathway is sufficient to induce the formation of margin bristles in the wing blade (Simpson et al., 1988; Blair, 1992; Diaz-Benjumea and Cohen, 1995). In the wild-type wing, bristles only form very close to the source of *wg* expression and so represent a response to high levels of Wg. MS1096:UAS*wg* induces wing margin bristles throughout the wing blade, indicating that MS1096:UAS*wg* produces high levels of *wg* activity (see Fig. 4.3.1D). However, MS1096:UAS*wg* does not cause an expansion of the expression of the early *vestigial* enhancer (Fig. 5.2.1H), indicating that *wg* is not

sufficient to direct expression of this enhancer. *Notch* activity is sufficient to do so when expressed under the same conditions (Fig. 5.2.1G; MS1096:UAS*Notch(intra)*). Similarly, misexpression of *Delta*, a ligand for Notch, on the dorsal side of the disc can direct ectopic expression of the early *vestigial* enhancer (Doherty et al., 1996), indicating that failure of ectopic Wg expression to direct expression of the early *vestigial* enhancer cannot be due to the absence of Notch.

To further clarify whether *wg* plays a role in *vestigial* expression at the D/V boundary, we produced clones of cells mutant for the null allele wg^{CX4}. Clones induced before the D/V compartmental restriction is established will remove *wg* on both sides of the D/V boundary, prior to the activation of the early *vestigial* enhancer by the D/V patterning system. Such clones cause extensive non-autonomous loss of wing tissue (Diaz-Benjumea and Cohen, 1995), but do not affect expression of the early *vestigial* enhancer (Fig. 5.3.1A,B). I also examined the expression of the early *vestigial* enhancer in $spade^{flag}$ (*spdfg*) mutant discs. Data presented in chapter 3 above indicate that *spdfg* reduces *wg* function in the wing margin in addition to the wing hinge. When *spdfg* is heterozygous with a deficiency that uncovers *wg*, anterior and posterior wing margin structures are absent with 100% penetrance. This correlates with an absence of detectable Wg protein in the anterior and posterior margin (Fig. 5.3.2B), and this phenotype can be rescued by expressing UAS*wg* at the D/V boundary (Fig. 3.2.5). Removing Wg activity from the anterior and posterior wing margins compromises expression of the *wg* target gene *Distal-less* in these regions (Fig. 5.3.2D), but has no effect on expression of the *vestigial* early enhancer (Fig. 5.3.2H). Furthermore, removing *wg* activity in late second instar using the wg^{ts} allele has no effect on the *vestigial* early enhancer (Fig. 6.2.1). Taken together with the observation that *wg* is not able to direct ectopic expression of the early *vestigial* enhancer, these results indicate that this enhancer does not

depend on *wg* signaling. Therefore, the early expression of *vestigial* is likely to be regulated by the *Notch* pathway alone.

To ask whether *wg* expression at the D/V boundary depends on the prior activation of *vestigial*, I examined *wg-lacZ* expression in wing discs homozygous mutant for the vestigial null allele (vg^{83b27R}; see Williams et al., 1993). In the absence of *vestigial* function, *wg* is initially activated at the D/V boundary, but then fades away (Fig. 5.3.3). While even the earliest expression of *wg* does not look entirely normal in *vestigial* null mutant discs, there is clear expression of *wg-lacZ* at the D/V boundary, suggesting that *vestigial* is not absolutely required for the activation of *wg*. It has been shown that there is extensive cell death in *vestigial* mutant discs, starting already in late second instar (Fristrom, 1969; James & Bryant, 1981). This suggests that the loss of *wg* expression in vestigial null mutant discs, as well as the slightly abnormal appearance of *wg* expression at earlier stages could be an indirect effect. This is supported by the observation that the expression of an *Enhancer of split m8-lacZ* construct is also lost at very early stages in vestigial null mutant discs (data not shown). The expression of this enhancer has been shown to be a direct target of the Notch pathway (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995), suggesting that its expression is lost due to a non-specific reduction of gene expression in the dying cells of the *vestigial* mutant wing pouch.

Figure 5.3.1: *vestigial* and *cut* expression in *wg* mutant clones

(A,B) *vestigial-lacZ* (red) is expressed normally in clones of cell mutant for the null allele wg^{cx4} (visualized by the absence of N-myc expression, green in merged image, single channel images shown below). Clones induced prior to activation of the D/V patterning system in the second instar disc can cross the D/V compartment boundary (arrow in A). Arrow in B indicates a large clone that removes Wg from the D/V boundary at the lateral edge of the wing pouch. Judging from the shape of the clone, this example may be two separate mutant clones that have merged at the boundary.
(C) *cut-lacZ* expression in wg^{cx4} mutant clones. Arrow at right indicates a dorsal clone that meets but does not cross the D/V boundary. *cut* expression is normal. Arrow at left indicates a clone that crosses the D/V boundary, and removes *cut* expression. (D) Detail of clone showing that *cut* is expressed in *wg* mutant cells at the edge of the clone, but expression is reduced in cells more distant from the edge.

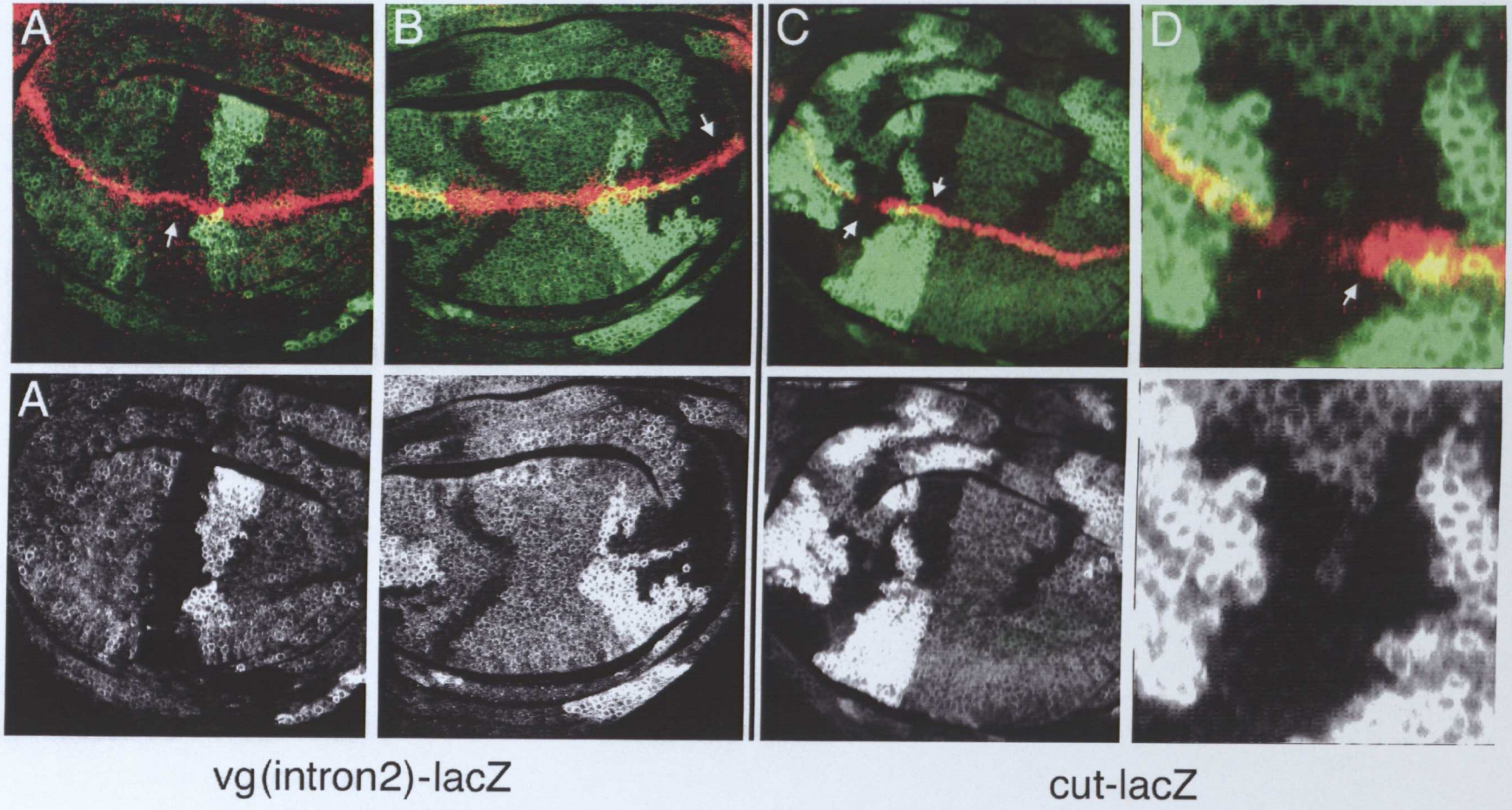

A
B
C
D
A
vg(intron2)-lacZ
cut-lacZ

Figure 5.3.2: *wingless* is required for cut, Distal-less and Vestigial expression, but not for the early *vestigial* enhancer.

(A, C, E, G, I) wild-type third instar wing discs. (B,D,F,H, J) *spdfg/Df(2L) hL2* wing discs. (A,B) Wg protein. (C, D) Distal-less protein. (E,F) *cut-lacZ.*. (G,H) *vestigial* early enhancer-lacZ. (I, J) Vestigial protein. Wg, Distal-less and cut expression are lost from the anterior and posterior margins of *spd* mutant discs (arrows). Expression of the *vestigial* intron 2 enhancer is normal (H), but expression of Vestigial protein is lost from the anterior and posterior regions of the wing pouch (arrow, J) except at the D/V boundary where the early enhancer is expressed.

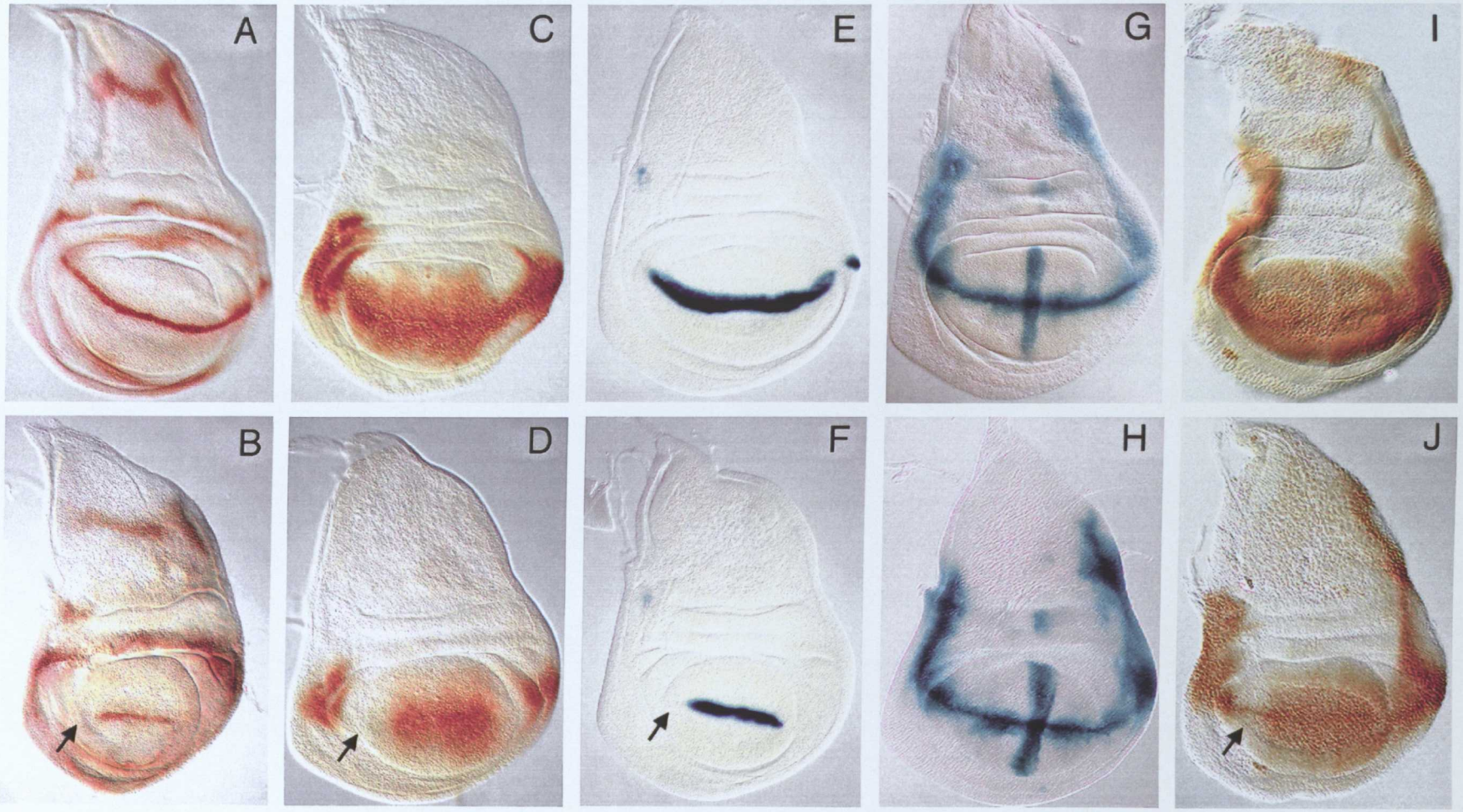

A
C
E
G
I
wild type
B
D
F
H
J
spd fg / Df(2L)hl2
Wingless
Distal-less
cut lacZ
vg (int2) lacZ
Vestigial

Figure 5.3.3: Vestigial activity is not required for the activation of Wingless.

(A-D) *wg-lacZ* expression detected with anti-β-galactosidase antibody. (A) wild-type late second instar wing disc. (B) vg^{83b27R} (null) mutant late second instar wing disc. Note that the activation of wg along the D/V boundary (arrowhead) appears to be normal. (C) wild-type early third instar wing disc. (D) vg^{83b27R} (null) mutant early third instar wing disc. The expression of *wg* along the D/V boundary is starting to be lost in patches (arrow). In later discs, no expression of *wg* at the margin can be detected, and *wg* expression in the hinge region is also often lost (data not shown). As the loss of wing tissue in *vestigial* mutants is at least partially due to extensive cell death, this may be an indirect effect.

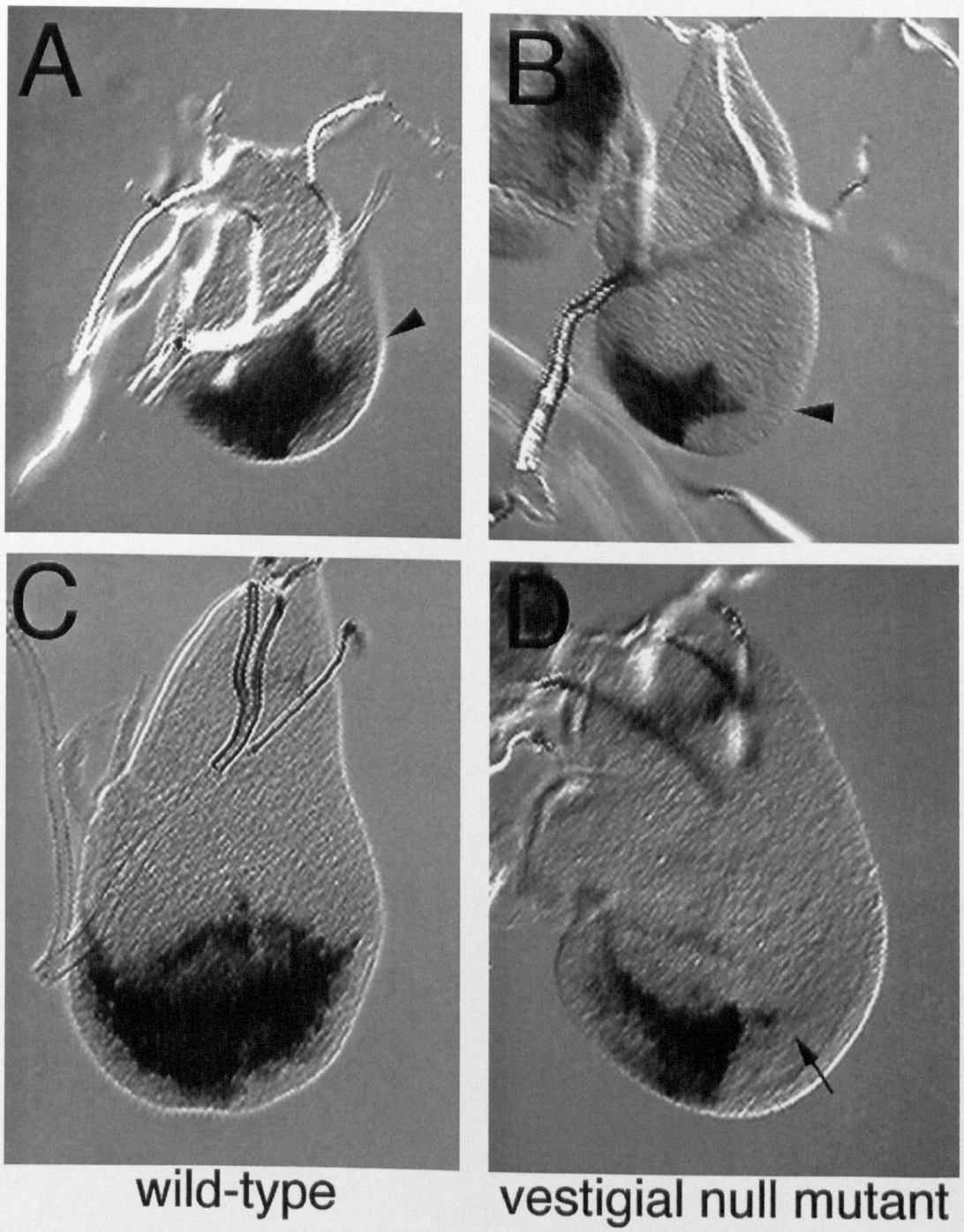
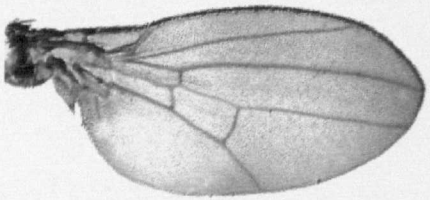

A
B
C
D
wild-type
vestigial null mutant

Figure 5.3.4: The vg^{83b27} phenotype can be rescued by *vg*GAL4:UAS*vg*, but not by *vg*GAL4:UAS*wg*.

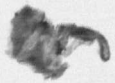
(A) Wing from an idividual of the genotype vg^{83b27} *vg*GAl4/vg^{83b27} UAS*vg*. Note the extensive rescue of the vg83b27 phenotype (compare with B). (B) Wing from an idividual of the genotype vg^{83b27}. (C) Wing from an individual of the genotype vg^{83b27} *vg*GAL4/vg^{83b27}; UAS*wg*/+. Note the absence of any rescue.

A



vg83b27 vgGAL4/vg83b27; UASvg

B



vg83b27

C

vg83b27 vgGAL4/vg83b27; UASwg

These results indicate that activity of *vestigial* is not required for the initiation of *wg* expression at the D/V boundary, and that *wg* and *vestigial* are parallel targets of the *Notch* pathway in these cells. Consistent with this proposal, it is not possible to rescue the phenotype of the *vestigial*^{83b27} regulatory mutant (which causes loss of vestigial expression at the D/V boundary) by expressing UAS*wg* under the control of the enhancer that is deleted in *vestigial*^{83b27} (Fig. 5.3.4). If the function of Vestigial were to activate *wg* at the D/V boundary, one would expect at least partial rescue (since UAS*vestigial* gives full rescue under the same conditions - see Fig. 5.3.4).

5.4: *wingless* and *Notch* cooperate to activate *cut*

The short-range patterning activities of the D/V boundary have been attributed to localized expression of *wg* (Phillips and Whittle, 1993; Blair, 1994; Couso et al., 1994; Rulifson and Blair, 1995). *wg* activity is required for specification of wing margin sense organs and for *cut* expression. *cut* is expressed in the same cells as *wg* at the D/V boundary, but unlike *wg*, *cut* is first activated in mid-third instar, about 24 hours after *wg* (Blair, 1993, 1994). Removing *wg* function during third instar using the wg^{ts} mutant causes loss of *cut* expression (Couso et al., 1994). Consistent with this result, Fig. 5.3.2F shows that *cut* expression is lost along the anterior and posterior wing margin of discs of the genotype $spdfg/Df(2L)spd^{hL2}$. To define the requirement for *wg* activity more precisely, I examined *cut-lacZ* expression in clones of *wg* mutant cells. *wg* mutant clones that touch the margin from one side only do not affect *cut* expression (Fig. 5.3.1C). Clones that cross the D/V boundary lose *cut* expression in the center, but retain *cut* in cells at the lateral edges of the clones (Fig. 5.3.1C,D). These data indicate that Wg can activate *cut* non-autonomously over short distances, but only in cells at the D/V boundary (Fig. 5.3.1D).

Although *wg* is required for *cut* expression, producing high levels of *wg* activity in the dorsal wing pouch using MS1096:UAS*wg* does not cause an expansion of *cut* expression (Fig. 5.2.1D). This indicates that Wg is not sufficient to direct *cut* expression and is consistent with the observation that clones of cells lacking activity of *shaggy/zeste-white3* (which is a negative regulator of the *wg* pathway) fail to activate *cut* (Blair, 1994).

These results indicate that Wg signaling can only direct *cut* expression in cells at the D/V boundary. Activity of the *Notch* pathway may be responsible for generating the competence of cells to activate *cut* in response to Wg. Removing activity of the *Notch* pathway in *Suppressor of*

Hairless clones causes cell-autonomous loss of *cut* expression. Even small *Suppressor of Hairless* clones of one to two cells lose *cut* expression (Fig. 5.2.2D). If the effect of *Notch* on *cut* were only indirect, via *wg*, then it would be expected that *cut* expression should be rescued non-autonomously in cells near the edge of a *Suppressor of Hairless* mutant clone, as in *wg* mutant clones (Fig. 5.3.1D). However, this is not the observed result. Taken together with the observation that MS1096:UAS*Notch(intra)* directs ectopic *cut* expression while MS1096:UAS*wg* does not (Fig. 5.2.1C,D), these data suggest that *Notch* activity is directly required for *cut* expression, and that the *wg* and *Notch* pathways synergize to activate *cut*.

5.5: Discussion

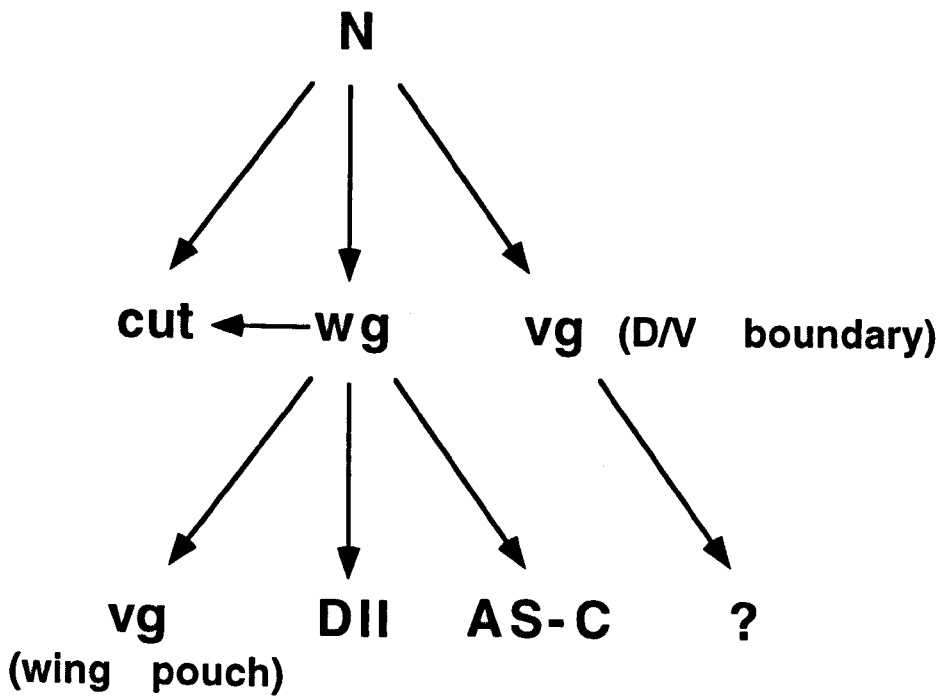
5.5.1: *wingless* and *vestigial* at the D/V boundary

The data presented above strongly suggest that *wg* and *vestigial* are parallel targets of the *Notch* pathway at the D/V boundary, and that *wg* plays no role in the activation of *vestigial* by *Notch* (Fig. 5.4.1). These results are incompatible with the proposal by Couso et al. (1995) that ventrally expressed Wg cooperates with dorsally expressed Serrate to induce expression of the *vestigial* early enhancer in cells straddling the D/V boundary of the wing disc. If this were the case, one would expect ectopic expression of Wg in dorsal cells (where Serrate is expressed) to direct ectopic expression of the *vestigial early* enhancer. However, this is not the observed result. One could argue that the MS1096 GAL4 driver is expressed too late, and that the cells have lost their competence to respond to Wg at this stage. However, the same result is observed if UAS*wg* is expressed under the control of the dppGAL4 driver, which is expressed already from very early larval stages (data not shown). In contrast, UAS*Delta* expressed under the control of dppGAL4 is able to induce the

expression of the *vestigial* early enhancer in dorsal cells (Doherty et al., 1996), strongly suggesting that the inability of ectopic Wg to activate this enhancer is not due to a loss of competence in the responding cells. The proposal that *wg* is required for the activation of the *vestigial* early enhancer is based on the observation that *vestigial* is lost in discs where *wg* activity is removed during second instar using the wg^{ts} allele (Williams et al., 1993; Couso et al., 1995). However, in the absence of *wg* activity during second instar cells fail to adopt wing identity and remain with the default identity of body wall (Morata and Lawrence, 1977; Couso et al., 1993; Ng et al., 1996). Thus the failure to specify the wing pouch precludes the activation of *vestigial* by the D/V system. Because this represents an earlier function of *wg*, it cannot be taken as evidence that *wg* is required to activate the *vestigial* early enhancer.

Consistent with the proposal that *wg*and *vestigial* are parallel targets of Notch at the D/V boundary, a Suppressor of Hairless binding site has been identified in the *vestigial* D/V boundary enhancer, and shown to be necessary for its acitivity (Kim et al., 1996). As it has also been shown that Notch signalling at the D/V boundary, unlike Notch signalling in most other contexts, does not depend on *Enhancer of Split* function (de Celis et al., 1996c), it is thus predicted that the activation of the *wg* D/V boundary enhancers similarly depends on direct binding of Suppressor of Hairless. Potential Suppressor of Hairless binding sites in the *spd* enhancer, which is expressed at the D/V boundary, are highlighted in Fig. 3.2.10. However, these sites do not conform exactly to the consensus (Lecourtois and Schweisguth, 1995), and it still remains to be shown that they are functional.

Figure 5.4.1: Schematic model of the genetic hierarchy downstream of Notch that establishes and mediates the function of the D/V organizer. Activated Notch establishes the organizer by directing expression of both *wingless* and the *vestigial* D/V boundary enhancer. Wingless then mediates short range effects of the organizer by activating *cut* and *achaete-scute complex* (AS-C) expression locally, and also long range effects of the organizer, by directing *vestigial* expression in a broad domain filling the wing pouch. Notch also has a direct function in the short range activity of the organizer by synergizing with Wingless to activate *cut*. The late activation of *cut* and the modest scalloping phenotypes of *cut* mutants suggest that it has a local function in wing margin differentiation. Apart from mediating the long range function of Wingless in the wing pouch, Vestigial also has a distinct and critical function in the cells of the D/V organizer, which is not yet clear.


N
cut
wg
vg (D/V boundary)
vg
(wing pouch)
DII
AS-C
?

5.5.2: What is the function of *vestigial* at the D/V boundary?

The answer to this question is not yet clear. *vestigial* activity is critically required for wing development; the wing and haltere are almost completely lost in *vestigial* mutants (Williams et al., 1993). *vestigial* appears to have at least two distinct functions; it is initially activated by *Notch* at the D/V boundary, and a regulatory mutant of *vestigial* that removes the early enhancer required for this activation causes a reduction of the wing that is as severe as that seen in a *vestigial* null mutant (Williams et al., 1994). Because *vestigial* encodes a nuclear protein, Vestigial expression in cells at the D/V boundary is unlikely to have a direct effect on cells at a distance, suggesting that *vestigial* must act upstream of another signal that relays the patterning information of the D/V organizer. I have presented evidence that this function is not mediated by *wg*, suggesting that there may be second signal in addition to *wg*, which depends on *vestigial* activity at the D/V boundary.

However, there may be an alternative possibility for the function of *vestigial* at the D/V boundary. It has been shown that *vestigial* mutant clones do not survive in the wing blade (Simpson et al., 1981; Kim et al., 1996), suggesting that *vestigial* is required for the growth or survival of cells in the wing. It has also been shown that ectopic expression of *vestigial* in other imaginal discs transforms cells to wing identity (Kim et al., 1996), suggesting that *vestigial* may have a function in specifying the identity of wing cells. It may be that this is also the function of Vestigial expressed at the D/V boundary. Removal of *vestigial* function from cells at the D/V boundary could lead to cell death, thereby eliminating the organizer cells and thus indirectly causing loss of the whole wing. However, Simpson et al. (1981) have shown that if *vestigial* mutant clones are given a growth advantage by using the minute technique (reviewed in Lawrence, 1992), then they

are able to survive and give rise to all pattern elements inside the wing blade, but they cannot contribute to the wing margin. Clones that touch the margin cause scalloping of the wing. This argues that the function of *vestigial* is different in the cells of the wing blade versus the cells at the D/V boundary. *vestigial* may have a growth/survival-promoting function in the cells of the wing blade and a more critical patterning function in the cells at the D/V boundary. Alternatively, the function of *vestigial* may be similar in both groups of cells, but the cells of the D/V boundary could be more sensitive to a reduction of *vestigial* activity.

5.5.3: *Notch* and *wingless* at the margin

Evidence for a negative cross-talk between the *Notch* and *wg* pathways has been presented by Axelrod and coworkers (1996), who suggest that this effect is mediated by Dishevelled protein binding to the intracellular domain of Notch. *dishevelled* is required to transduce the *wg* signal in the embryo and in the imaginal discs (Couso et al., 1994; Klingensmith et al., 1994; Nordermeer et al., 1994; Siegfried et al., 1994; Theisen et al., 1994). Increasing the level of *dsh* expression can produce phenotypes similar to those caused by ectopic *wg* expression (see section 4.3 above; Yanagawa et al., 1995; Axelrod et al., 1996). A reduction in the activity of *Notch* or *Delta* potentiates this effect, while overexpression of the Dishevelled-binding site in Notch inhibits it, presumably by titrating out Dishevelled (Axelrod et al., 1996). These observations are consistent with a model in which the *Notch* and *wg* pathways antagonize each other in regulation of the *achaete-scute* complex and neural cell fate determination. Rulifson et al. (1996) have also presented data suggesting that Wg is required to repress its own *Notch*-dependent expression in cells outside of the D/V boundary domain.

I have presented evidence that the *Notch* and *wg* pathways can also act co-operatively (Fig. 5.4.1). The observation that Wg can only activate *cut* in cells at the D/V boundary, and that *Suppressor of Hairless* is required cell autonomously for *cut* expression suggests that activity of both pathways is required in the cells abutting the D/V boundary to activate *cut* expression. How can Notch and Wg act antagonistically to regulate *achaete-scute complex* expression in cells near the D/V boundary while simultaneously acting synergistically on *cut* expression in cells at the D/V boundary? One possibility is that Notch might be required for the reception of the Wg signal (Couso & Martinez Arias, 1994). This seems unlikely because *Notch* is not needed for the reception of the Wg signal in specification of sense organ precursors (Rulifson and Blair, 1995), nor is *Suppressor of Hairless* required for the expression of other *wg* targets in the wing disc (data not shown). Also, ectopic expression of the *Notch*-ligands *Serrate* or *Delta* is able to activate the *Notch* pathway in the wing disc, while ectopic expression of *wg* is not.

An alternative explanation for the simultaneous co-operation and mutual inhibition between Notch and Wg may be that the observed high levels of Serrate and Delta in the cells adjacent to the D/V boundary (Kim et al., 1995; Doherty et al., 1996) keep Notch in the activated state in these cells and make it insensitive to inhibition by Dishevelled. This could allow simultaneous activity of the *Notch* and *wg* pathways in the cells abutting the D/V boundary, while in the cells that are a few cell diameters away from the D/V boundary, the absence of high levels of Serrate and Delta would allow secreted Wg to inhibit the *Notch* pathway. This view is consistent with the model proposed by Rulifson et al. (1996). These authors show that secreted Wg inhibits its own expression in cells close to the D/V boundary, most likely by inhibiting Notch activity, but also propose that this inhibitory effect is not strong enough to overcome the high level of Notch activity in the cells directly abutting the D/V boundary.

CHAPTER 6:
LONG-RANGE SIGNALLING BY WINGLESS IN A CONCENTRATION-DEPENDENT MANNER MEDIATES THE ORGANIZING ACITVITY OF THE D/V ORGANIZER

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6.1: Introduction

The long-range patterning activities of the D/V organizer have been attributed to localized expression of *wg*, because removal of *wg* activity (after the wing has been specified in second instar) results in extensive loss of tissue from the outer edge of the wing (Diaz-Benjumea and Cohen, 1995; see also Figs. 6.5.1 and 6.5.2 below), and because clones of Wg-expressing cells produce outgrowths from the wing surface that resemble the axis bifurcations caused by producing an ectopic D/V boundary (Diaz-Benjumea and Cohen, 1995; see also Fig. 6.3.3 below). Wg protein is secreted (van Leeuwen et al., 1994) and can be detected in vesicular structures in the cytoplasm of nearby cells (van den Heuvel et al., 1989; Gonzalez et al., 1991). This raises the possibility that secreted Wg protein may directly mediate the long-range patterning and growth-promoting activities of the D/V boundary. To address this question, it is necessary to identify target genes of *wg* signaling in the wing pouch, and to analyze their regulation by *wg*.

In this chapter, I present data suggesting that Wingless acts directly, at long range, to define the expression domains of its target genes, *Distal-less* and *vestigial*. Expression of the *Acheate-scute* genes, *Distal-less* and *vestigial* at different distances from the D/V boundary appears to be controlled by Wingless in a concentration-dependent manner, suggesting that Wingless functions as a morphogen in patterning the D/V axis of the wing.

6.2: *wingless*, expressed at the D/V boundary, directs expression of *vestigial* and *Distal-less* in broad domains in the wing pouch

It has been suggested that the D/V system works through *vestigial* to control growth of the wing (Williams et al., 1994; Kim et al., 1996). Vestigial encodes a novel nuclear protein and is expressed in a broad domain thoughout the wing pouch, which is centered on the D/V boundary. Only a subset of this expression domain is under the control of the early enhancer. Expression of the early enhancer does not depend on *wg* activity (see section 5.3 above); however, removing *wg* activity in late second instar using the wg^{ts} mutant leads to complete loss of the secondary expression of Vestigial in the wing pouch without affecting expression at the D/V boundary, where the early enhancer is active (Fig. 6.2.1E). Removing *wg* activity later, in third instar, still strongly reduces Vestigial expression in the wing pouch (Fig. 6.2.1B,C), suggesting that *wg* signalling is required both to initiate and to maintain *vestigial* expression. A comparable result is obtained when *wg* is removed locally from the anterior and posterior wing margins; Vestigial expression is normal at the margin, but is reduced in the adjacent regions of the wing pouch (Fig. 5.3.2J).

These results indicate that localized expression of *wg* at the D/V boundary is required for Vestigial expression throughout the wing pouch. *wg* does not regulate the early D/V boundary enhancer of *vestigial*, but instead appears to regulate *vestigial* through the recently described "quadrant enhancer" that drives expression in the rest of the wing pouch (Kim et al., 1996). Ectopic expression of UAS*wg* under the control of *dpp*GAL4 causes the elevation of quadrant enhancer-*lacZ* expression, while the reduction of *wg* activity in wg^{ts} discs causes the loss of quadrant enhancer-*lacZ* expression (Fig. 6.2.2).

Figure 6.2.1: Wingless activity is required for Vestigial expression in the wing pouch, but not at the wing margin.

(A-C) Mature third instar wing disc stained for Vestigial protein. (A) $wg^{IL114}/+$ disc. Note the broad domain of Vestigial expression filling the wing pouch (arrow)
(B, C)wg^{IL114} discs. Wg activity was removed in early third instar. Vestigial expression at the D/V boundary is not affected, but is reduced in the wing pouch (arrows). The disc in (C) is more severely affected than the disc in (B), and may have been slightly younger when Wg was inactivated.
(D,E) Mid-third instar wing discs stained for Vestigial. (D) $wg^{IL114}/+$. Note the high level of Vestigial at the D/V boundary and the lower level in the wing pouch. Expression at the D/V boundary is controlled by the early enhancer. (E) wg^{IL114} disc. Wg activity was removed in late second instar. Vestigial expression at the D/V boundary is not affected, but is lost in the wing pouch.

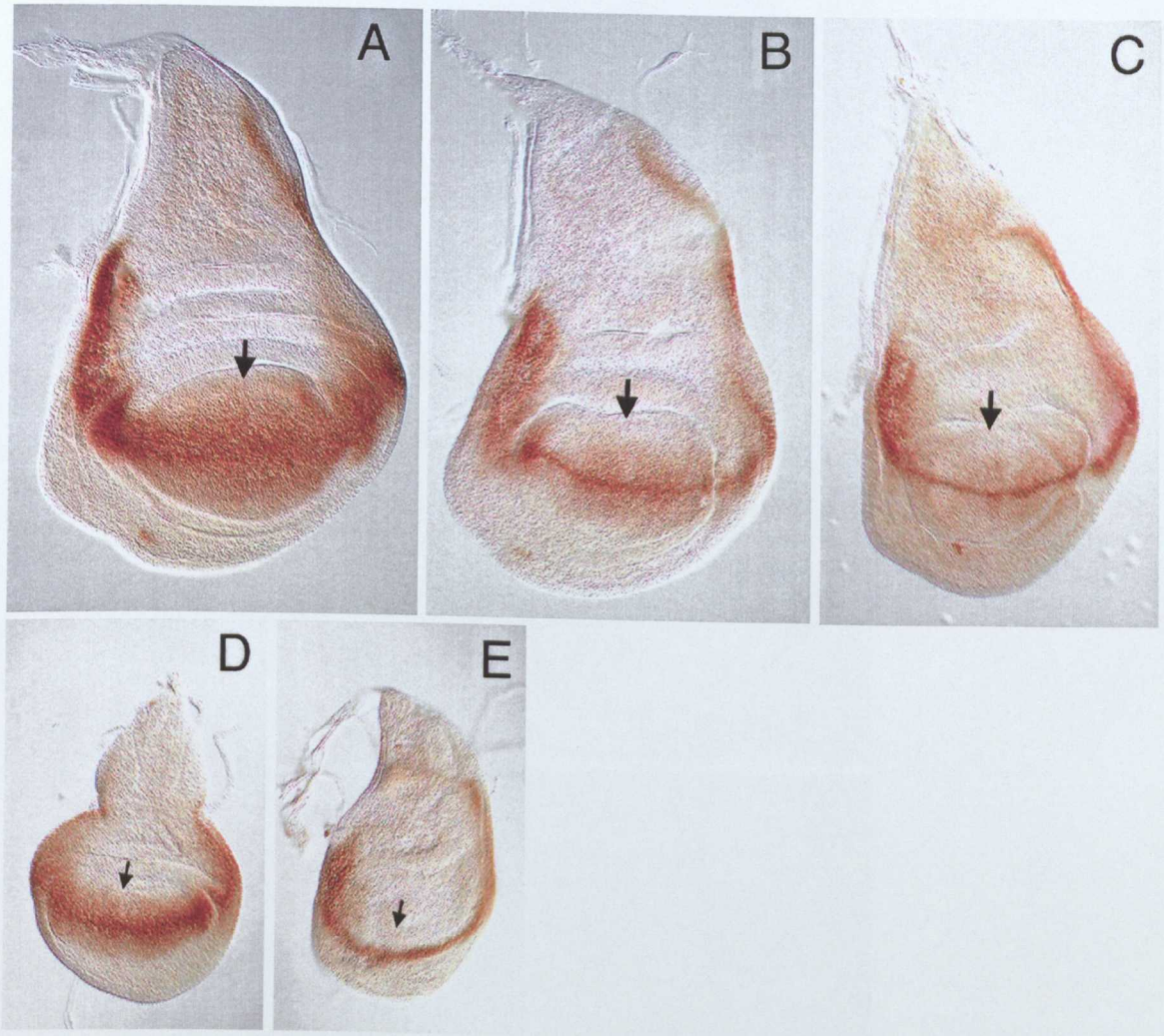

A
B
C
D
E

Figure 6.2.2: Wingless regulates *vestigial* expression through the 'quadrant' enhancer.

A - C: X-gal stain of the *vg* quadrant-enhancer-*lac-Z* reporter gene. (A) *dpp*GAL4:UAS*wg*. (B) Wild-type. (C) wg^{IL114} at 24°C.
Note the overgrowth in A, and the elevation of quadrant-enhancer lacZ expression. Note also the strong reduction of quadrant-enhancer-lacZ expression in C.

A
B
C

Figure 6.2.3: Localized expression of Wingless is sufficient to activate *vestigial* in a broad domain in the wing pouch.

A, B: Wg protein. C: X-gal stain. D, E, F: Vg protein.
(A, D) wild-type. (B, E) *spdfg Sp/wg*^{CX4}. (C) *dpp*GAL4:UAS*lacZ*.
(F) *spdfg Sp/wg*^{CX4}; *dpp*GAL4:UAS*wg*.
Note that Vg expression is strongly reduced in E, and that it is activated in a broad domain centered on the A/P boundary in F.

A
Wg
B
C
D
Vg
wild-type
E
spdfgSp/wgCX4
F
spdfgSp/wgCX4;
dppGAL4/UASwg

Since Vestigial is normally expressed throughout the wing pouch, and cells outside of the wing pouch are unable to activate vestigial in response to Wingless, it is difficult to ask whether Wingless is not only necessary, but also sufficient to activate Vestigial. Therefore, I expressed *dpp*GAl4:UAS*wg* in a *spdf8Sp/wg*^{CX4} mutant background. Under these conditions, the broad domain of Vestigial, which normally straddles the D/V boundary, is rotated by 90^{o} and instead straddles the A/P boundary (Fig. 6.2.3), indicating that localized expression of Wingless is indeed sufficient to activate vestigial in a broad domain in the wing pouch.

Distal-less encodes a homeodomain transcription factor that is expressed in a broad domain in the wing pouch similar to Vestigial (Figs. 5.3.2 and 6.4.1). Although the maximal extent of the Distal-less domain is similar to that of Vestigial, Distal-less expression is more graded, and decreases from high levels at the D/V boundary to very low levels near the edge of the wing pouch (Fig. 6.4.1A). Removal of *wg* activity in late second instar using the *wg*^{ts} mutant leads to complete loss of Distal-less expression from the wing imaginal disc (Fig. 6.4.1C). Also, localized removal of *wg* activity from the anterior and posterior wing margins causes the loss of Distal-less expression in these regions (Fig. 5.3.2D). These results indicate that *Distal-less*, like *vestigial*, is a target of *wg* signaling in the wing imaginal disc. But unlike *vestigial*, all *Distal-less* expression appears to depend on *wg*. There is no *Notch*-dependent expression at the D/V boundary.

Several published studies have shown that *wg* expression in the wing pouch is both necessary and sufficient for the specification of wing margin bristles (Simpson et al., 1988; Blair, 1992; Phillips and Whittle, 1993; Couso et al., 1994; Diaz-Benjumea and Cohen, 1995), and that *wg* regulates bristle specification through the activation of the genes of the *acheate-scute complex* (Blair, 1992; Couso et al., 1994; Rulifson et al., 1996). Taken together, these results suggest that the

acheate-scute complex, *Distal-less*, and *vestigial* are targets of *wg* signalling in the wing pouch.

6.3: Activation of *vestigial* and *Distal-less* by *wingless* is direct and at a distance

To ask whether *wg* signaling directly defines the spatial domains of *vestigial* and *Distal-less* expression I examined clones of cells mutant for a component of the *wg* signal transduction pathway, *armadillo* (Peifer et al., 1991; Siegfried et al., 1994; Nordermeer et al., 1994). Large clones of cells mutant for strong *armadillo* alleles cannot be recovered, especially in regions where *wg* signaling is required (Peifer et al., 1991). To circumvent this problem, I made clones of cells mutant for the temperature sensitive hypomorphic allele $armadillo^{H8.6}$ (Klingensmith et al., 1989). Armadillo is the homologue of vertebrate β-catenin, a component of adherens junctions (Peifer, 1993). Sanson et al. (1996) have presented data suggesting that the different roles of Armadillo in cadherin-mediated cell adhesion and in *wg* signal transduction are functionally seperate and can be uncoupled from each other. Consistent with this proposal, Orsulic and Peifer (1996) have shown that several domains of Armadillo are specific for the function in cell adhesion, while others are specific for the function in *wg* signal transduction. These authors also show that the mutation $armadillo^{H8.6}$ only impairs the function of Armadillo in *wg* signaling, but not the function of Armadillo in cell adhesion. Therefore, the $armadillo^{H8.6}$ mutation is very useful to study the role of *wg* signaling in the regulation of *vestigial* and *Distal-less* expression in the wing pouch.

Figure 6.3.1: Cell-autonomous loss of Vestigial and Distal-less expression in *arm* mutant clones.

(A) Clone of cells homozygous for $arm^{H8.6}$ shifted to the non-permissive temperature in mid third instar, 18 hours before staining. The clone is marked by the absence of N-myc expression (green in A,B,D, single channels below). Note the absence of Vg expression in the clone (red in A, single channel below).
(B) Clone of cells homozygous for $arm^{H8.6}$, treated and stained as in A. Note that in this case Vg expression is only partially lost. The clone is outlined in B.
(C) Clone of cells homozygous for $arm^{H8.6}$, treated as in A and double labeled for Vg and Dll expression (Dll in green). In this case, absence of Vg expression was used to mark the clone. Note that the cells in which Vg is reduced, lack Dll expression.
(D) Clones of cells homozygous for $arm^{H8.6}$, treated as in A and double labeled for N-myc (green) and Engrailed (red). Engrailed expression is not affected in the clones.

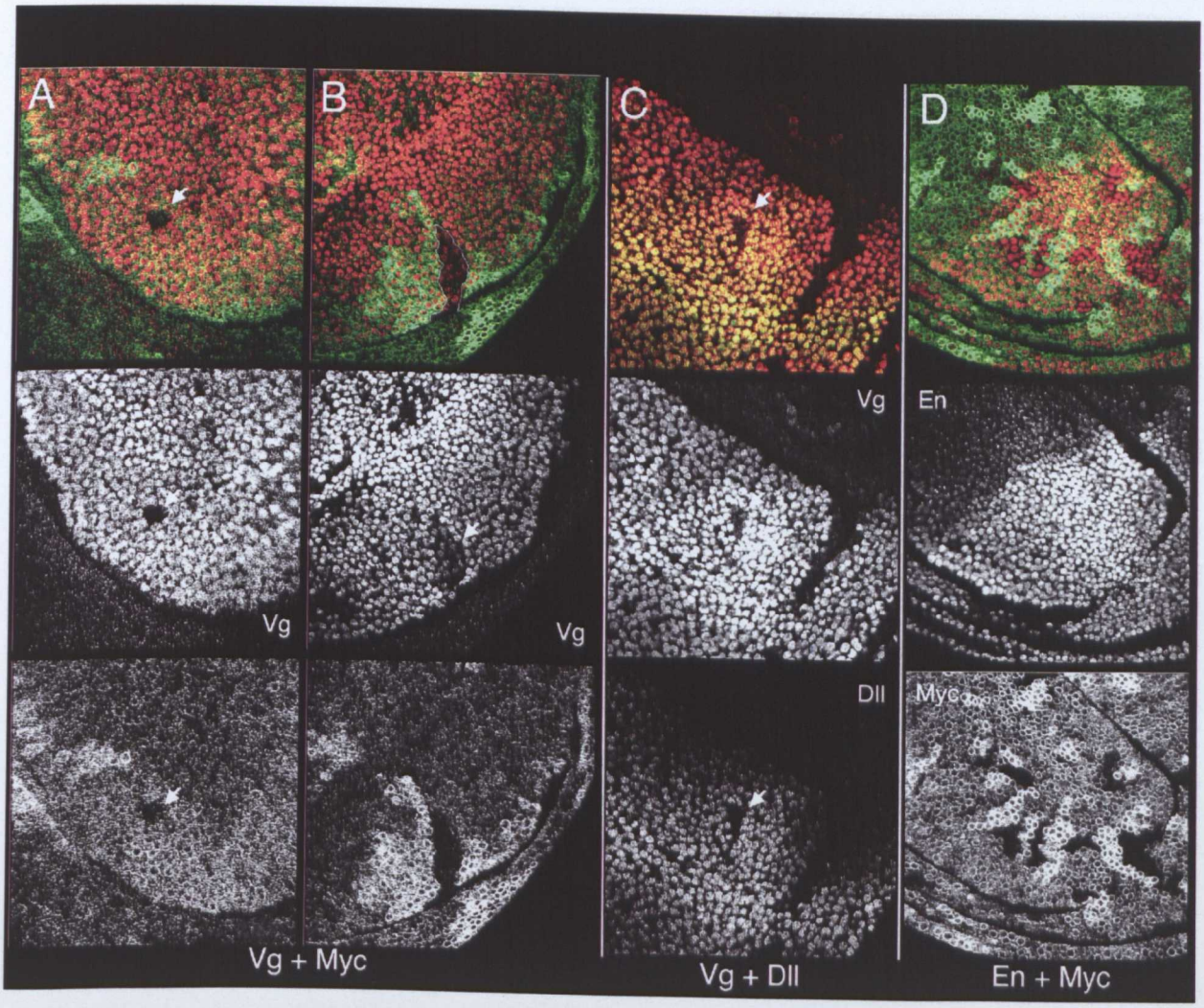

A
B
C
D
Vg
Vg
Vg
En
DII
Myc
Vg + Myc
Vg + DII
En + Myc

Figure 6.3.2: *armadillo* mutant clones survive in the notum, but not in the wing pouch.

Two examples of $arm^{H8.6}$ clones that were shifted to the nonpermissive temperature 48 hours before staining. Note that clones can be recovered in the notum and the hinge region of the discs, but not in the wing pouch region, while twin spots are found in both areas.

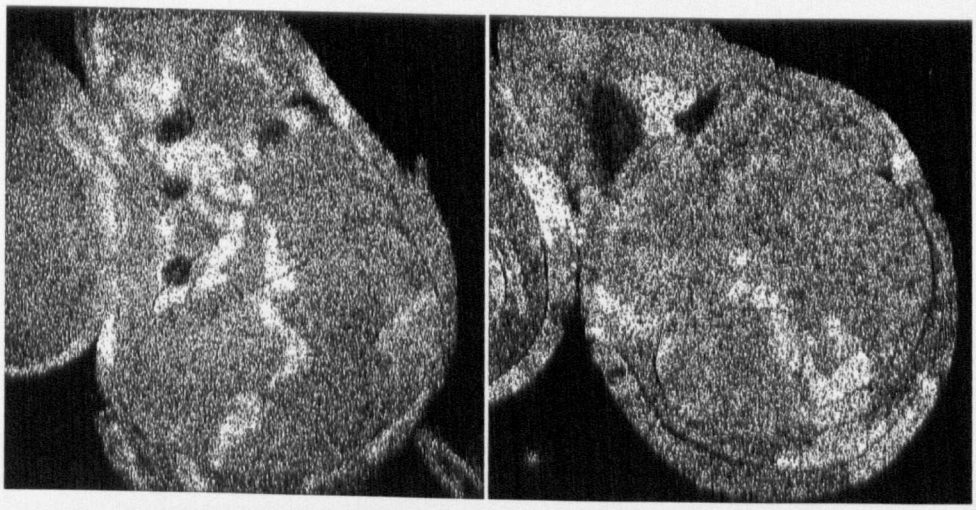


Table 6.1: Differential recovery of *armadillo* mutant clones in wing and body wall

	mutant clones	wild-type twins
wing pouch	3 (*)	84
notum	76	91

arm$^{H8.6}$ clones were induced in early second instar and allowed to grow at 17.5°C until early third instar. Larvae were then shifted to 25°C to reduce Arm protein activity for 48 hours. Mutant clones and wild-type twin spots were counted in the wing pouch and notum in 34 imaginal discs. (*) The three clones recovered in the wing pouch were very small.

armadillo$^{H8.6}$ clones were induced and larvae were raised at the permissive temperature (17.5°C) to allow Armadillo to function while the clones grow. Larvae were then shifted to 25°C to reduce Armadillo protein activity at different times before the end of third instar. Mutant clones could be recovered at reasonable frequency in the wing pouch of discs shifted to the restrictive temperature 18 hours before the end of 3rd instar (Fig. 6.3.1), but not in discs shifted 48 hours before the end of 3rd instar (Table 2 and Fig. 6.3.2). Mutant clones were recovered in the portion of the disc that gives rise to the dorsal thorax, or notum, under both conditions. After 18 hours at 25°C, clones in the wing pouch have either lost Vestigial expression (Fig. 6.3.1A) or show reduced levels of Vestigial expression (Fig. 6.3.1B). Distal-less expression is also lost in *armadillo* mutant clones (Fig. 6.3.1C). Both effects are cell autonomous. To be certain that the effect on Vestigial and Distal-less expression is specific, and does not reflect a general loss of gene expression, I verified that Engrailed expression is unaffected in *armadillo*$^{H8.6}$ mutant clones under the same conditions (Fig. 6.3.1D).

Another way to ask whether *vestigial* and *Distal-less* are direct targets of the *wg* signaling pathway is to examine the effect of cell-autonomously activating the *wg* pathway in a group of cells. Misexpression of Wg under the control of dppGAL4 generates a second source of Wg in cells along the A/P compartment boundary, along a line perpendicular to the endogenous source at the D/V boundary. Under these conditions, Vestigial and Distal-less expression is elevated in broad domains centered on the A/P boundary (Fig. 6.3.3A). Although dppGAL4:UAS*wg* is only expressed in anterior cells, ectopic expression of both target genes is also seen in the posterior compartment. This is evident when compared to the effect of cell-autonomous activation of the *wg* pathway by over-expression of UAS*dishevelled* by dppGAL4, which results in increased expression of Vestigial and Distal-less only in anterior cells (Fig. 6.3.3B,C).

Figure 6.3.3: Non-autonomous activation of Vestigial and Distal-less by Wg, but not by Dsh.

(A) *dpp*GAL4:UAS*wg* wing disc. Vg in red, Dll in green (single channels below). *dpp*GAL4 is expressed along the anterior side of the AP boundary. Note high levels of Vg and Dll expression along the A/P compartment boundary (arrow), and the graded expression to either side. Note also the overgrowth of the disc along the AP axis.
(B) *dpp*GAL4:UAS*dsh* wing disc. Channels as in A. Note elevation of Vg and Dll in the same cells along the AP boundary (arrow). Note also the lack of overgrowth.
(C) *dpp*GAL4:UAS*dsh* wing disc, double labeled for Ci (red) and Dll (green). Ci is expressed only in anterior cells. Note that Dll is only elevated in a stripe of cells to the anterior of the compartment boundary, corresponding to the cells in which the *dpp*GAL4 driver is expressed. The image in panel C is magnified ~1.6 fold compared to panels A, B and D)
(D) Wild-type disc. Staining as in A and B.

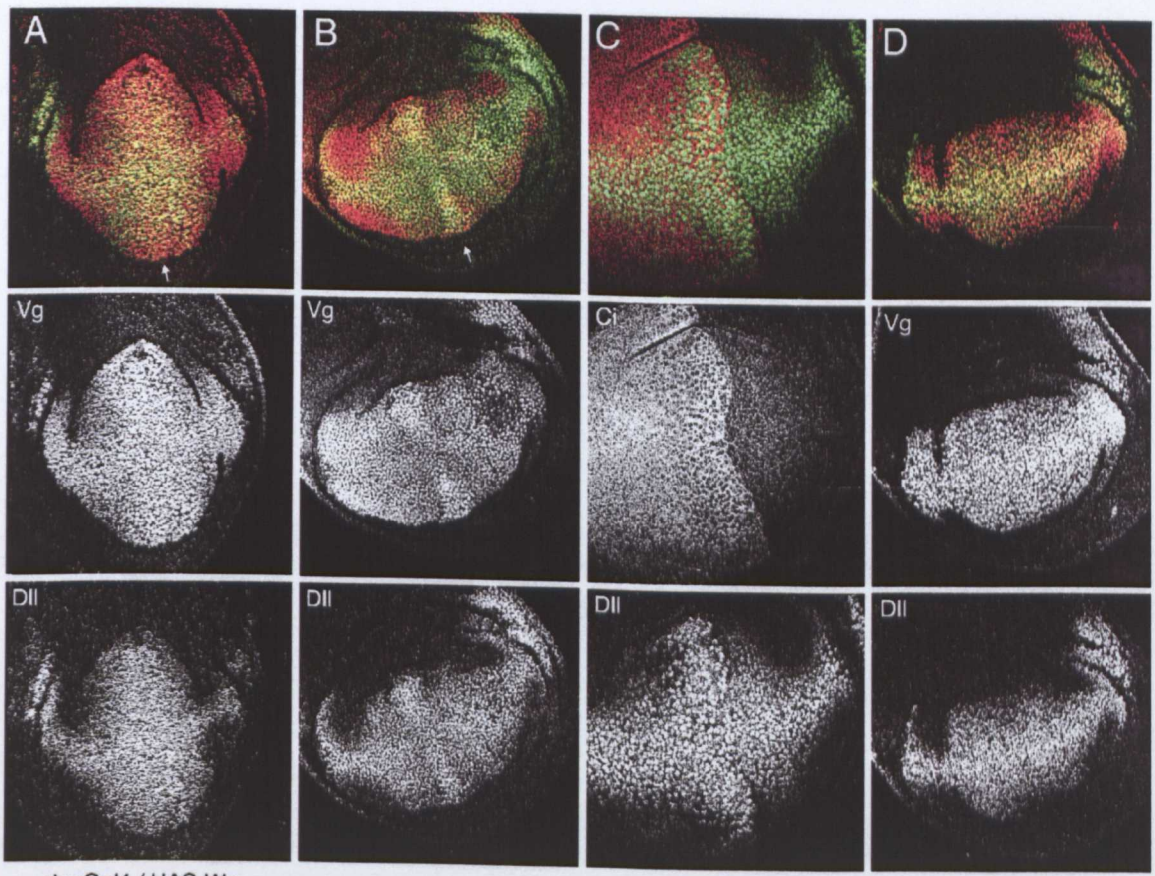
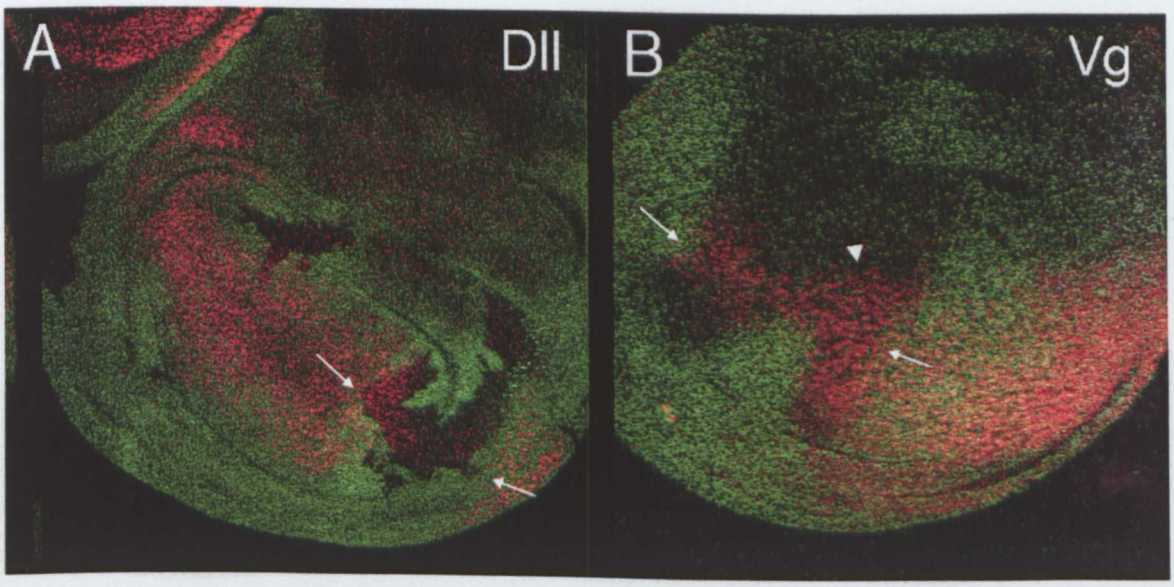

A
B
C
D
Vg
Vg
Ci
Vg
Dll
Dll
Dll
Dll
dppGal4 / UAS-Wg
dppGal4 / UAS-Dsh
wild type

Figure 6.3.4: Reduction of Vestigial and Distal-less expression due to large *wg* mutant clones

(A) Distal-less protein (red) expression in a disc with a large wg^{CX4} (null) mutant clone that crosses the D/V boundary (marked by the absence of *myc* staining (green) (B) Vestigial protein expression (red) in a disc with a large wg^{CX4} mutant clone that crossed the D/V boundary. In both discs, the D/V boundary is marked at the clone boundaries. Note that in both cases, gene expression is reduced inside the clone, but that there is also long-range non-autonomous rescue of expression in cells that are close enough to wild-type Wg-secreting cells. The range of this rescue is longest for Vestigial (arrowhead in B); compare this to the modest range over which *cut* expression is rescued inside wg mutant clones (Fig. 5.3.1).


A
Dll
B
Vg

Double-labeling for Distal-less and Cubitus interuptus protein (a marker for the anterior compartment) shows that Distal-less is not mis-expressed in posterior cells under these conditions (Fig. 6.3.3C). The domain of Distal-less has a sharp posterior border, unlike the case when dppGAL4:UAS*wg* is expressed. Double-labeling of Vestigial and Distal-less protein shows that both proteins are over-expressed in the same cells (Fig. 6.3.3B). These results are consistent with the observation that both Vestigial protein and Distal-less protein are cell-autonomously over-expressed in *shaggy/zeste-white3* mutant clones located in the wing pouch (Blair, 1994; Diaz-Benjumea and Cohen, 1995). *shaggy/zeste-white3* is a negative regulator of the *wg* pathway (Siegfried et al., 1992).

It has also been previously shown that *shaggy/zeste-white3* mutant clones autonomously activate expression of the *acheate-scute complex* (Blair, 1992), and that *dishevelled* mutant clones autonomously lose expression of the *acheate-scute complex* genes (Rulifson et al., 1996).

These observations indicate that secreted Wg protein acts directly to activate the expression of *vestigial*, *Distal-less* and the *acheate-scute complex* genes in the wing pouch. Because the pattern of Wg expression is dynamic in the wing disc, it is necessary to ask whether the localized expression of Wg in cells at the D/V boundary is directly responsible for this long-range activity. In this context it is important to point out that Wg is transiently expressed at low levels in all cells of the wing pouch in the early third larval instar (Couso et al., 1993; Phillips and Whittle, 1993; Ng et al., 1996), so it is formally possible that this domain of Wg expression could be responsible for activating *vestigial* and *Distal-less*. However, clones of cells mutant for a null allele of *wg* (wg^{CX4}) that meet the D/V boundary from either side or that occupy large regions of the wing pouch without meeting the D/V boundary have no effect on either Vestigial or Distal-less expression (data not shown) and do not cause loss of the wing margin or scalloping of wing blade tissue (Diaz-Benjumea and Cohen,

1995). Loss of wing tissue and of Vestigial and Distal-less expression is only observed when mutant clones eliminate *wg* in large stretches of cells on both sides of the D/V boundary (Diaz-Benjumea and Cohen, 1995; Fig. 6.3.4). Also, the ability to receive the *wg* signal in order to express Vestigial and Distal-less at a distance from the D/V boundary is still required at late stages, when Wg expression has retracted to the D/V boundary (see above; Fig. 6.3.1). These observations effectively rule out the possibility that the transient domain of *wg* expression throughout the wing pouch in early third instar is responsible for the broad expression of *vestigial* and *Distal-less*. Instead, they indicate that Wg secreted from the cells of the D/V boundary is directly responsible for the activation of Vestigial and Distal-less throughout the wing pouch.

6.4: Two distinct thresholds of Wingless signaling define the expression domains of the *acheate-scute complex*, *Distal-less* and *vestigial*

Taken together, the results presented in the previous section suggest that the genes *vestigial*, *Distal-less*, and the genes of the *acheate-scute complex* are direct targets of the *wg* signalling pathway. *acheate-scute* genes are expressed immediately adjacent to the D/V boundary (Blair, 1992). *Distal-less* expression straddles the D/V boundary and extends 15-20 cells into the wing pouch (Fig. 6.4.1A). *vestigial* expression also extends 15-20 cells into the wing pouch, but is stronger than *Distal-less* expression at the edges of the wing pouch, and appears to extend a few cells further than *Distal-less* in some areas (Fig. 6.4.1A). How does Wg specify these different expression domains? One possibility is that the secreted Wg protein forms a concentration gradient with a peak at its source at the D/V boundary, and that this gradient acts in a concentration-dependent manner to activate target

genes at different distances, because these target genes have different activation thresholds.

To address the issue whether Wg might be acting on its different target genes in a concentration-dependent manner, I examined the effect of partially reducing *wg* function using the conditional wg^{ts} mutant. In wg^{ts} mutant discs shifted to 22°C (a temperature that reduces *wg* activity to intermediate levels), the wing pouch is reduced in size (Fig. 6.4.1B). The domains of Vestigial and Distal-less expression are narrowed relative to a wild-type control (compare Fig. 6.4.1B with 6.4.1A). *A101* (*neuralized-lacZ*) is expressed in neuronal cells, and can thus be used as a marker for the bristle precursor cells of the anterior wing margin. In wg^{ts} mutant discs raised at 22°C, no *A101* expression can be detected at the D/V boundary (Fig. 6.4.1E), suggesting that *wg* activity has fallen below the level required for the activation of *acheate-scute* expression. wg^{ts} mutants raised at 22°C die at pharate pupal stages, making it possible to dissect out the wings produced by these individuals. These wings are greatly reduced in size compared to wild-type controls, and have no wing margin (Fig. 6.4.1F), confirming that *wg* activity has fallen below the level required for the specification of wing margin cell fates. Since Vestigial and Distal-less are still expressed (albeit at reduced levels) in these discs (Fig. 6.4.1B), this result indicates that a higher threshold of *wg* signaling is required for the activation of *acheate-scute* expression than for *vestigial* and *Distal-less* expression.

In wild-type discs Distal-less expression fades out at the edge of the wing pouch, while Vestigial expression remains relatively strong even in these cells (Fig. 6.4.1A), suggesting that *vestigial* may be more sensitive to low levels of Wg than *Distal-less*. In wg^{ts} mutant discs raised at 24°C (a temperature that strongly reduces, but does not eliminate wg^{ts} activity), Distal-less expression is completely lost from the wing pouch, but a low level of *wg*-dependent Vestigial expression can still be seen in the wing pouch (Fig. 6.4.1C, arrow). This result

suggests that the level of *wg* activity minimally required to activate *Distal-less* is higher than that required to activate *vestigial*. At 25°C, all *wg*-dependent Vestigial expression in the wing pouch is also lost (Fig. 6.2.1E).

The effect of *dishevelled* mutant clones on the expression of the three target genes of *wg* in the wing pouch further suggests that Wg regulates the spatial domains of its target genes in a concentration-dependent manner. *dishevelled* is a component of the *wg* signal transduction pathway (Theisen et al., 1994; Klingensmith et al., 1994). Cells mutant for *dishevelled* show reduced levels of Distal-less expression (Fig. 6.4.2C). Distal-less levels are reduced near the D/V boundary, where Wg levels are exprected to be high, but are completely lost further away from the D/V boundary, where Wg levels are expected to be lower, with the result that Distal-less expression is restricted to a narrower region of the wing pouch within the clone than in adjacent wild-type cells. The Distal-less levels at the D/V boundary inside the clone are similar to those located at a greater distance from the D/V boundary in the neighbouring wild-type cells. Vestigial expression is also reduced in *dishevelled* mutant clones near the edge of the wing pouch, in the cells farthest away from the D/V boundary (Fig. 6.4.2A,B). However, unlike Distal-less, Vestigial expression does not completely disappear in these cells, and is unaffected close to the D/V boundary. The finding that Distal-less expression is more strongly reduced in *dishevelled* clones than Vestigial, is consistent with the proposal that a higher level of *wg* signalling is required to activate *Distal-less* than is needed to activate *vestigial*. The effects on Vestigial and Distal-less expression are autonomous to mutant cells.

Figure 6.4.1: Two concentration thresholds for Wg signaling in the wing pouch.

(A) Wild-type. Dll expression is shown in green, Vg in red. The single channels are shown to the right. Vg expression extends throughout the wing pouch, and is slightly graded (see Williams, et al. 1994). Dll expression is strongest near the DV boundary and can be detected at lower levels toward the edges of the wing pouch. The gradient of Dll expression appears to be steeper than that of Vg expression.
(B) *wg^{IL114}* disc shifted to 22° in early third instar to reduce Wg activity to an intermediate level. Dll expression (green) is lost from the base of the wing pouch, but is still present in the center, although it is weaker and narrower than in wild-type. Vg expression (red) is also reduced and contracted in its spatial extent, but still occupies more territory than Dll (compare the expression of the two genes between the arrows). Vg expression at the DV boundary is unaffected. Single channels are shown to the right, as in A.
(C) *wg^{IL114}* disc shifted to 24° in early third instar to strongly reduce, but not eliminate Wg activity. Vg expression (red) can still be detected at low levels in the reduced wing pouch (arrow), but Dll expression (green) is not detectable. The single channels are shown to the right, as in A.
(D) Wild-type. *A101/neuralized-lacZ* expression (red) marks the rows of neuronal cells that give rise to the anterior wing margin bristles (arrow). Dll expression in green.
(E) *wg^{IL114}* disc shifted to 22° in early third instar. *A101* expressing cells at the D/V boundary are completely lost (arrow), while there is still some Dll expression (green). Note that Dll expression in this disc is reduced somewhat more than in the disc in panel B.
(F) Wing from a *wg^{IL114}* individual shifted to 22° in early third instar. The wing was dissected out of the pupal case and inflated, resulting in a somewhat damaged appearance (these larvae don't survive to adulthood). The wing margin is absent. These wings are of similar size to the one shown in Fig. 2B.

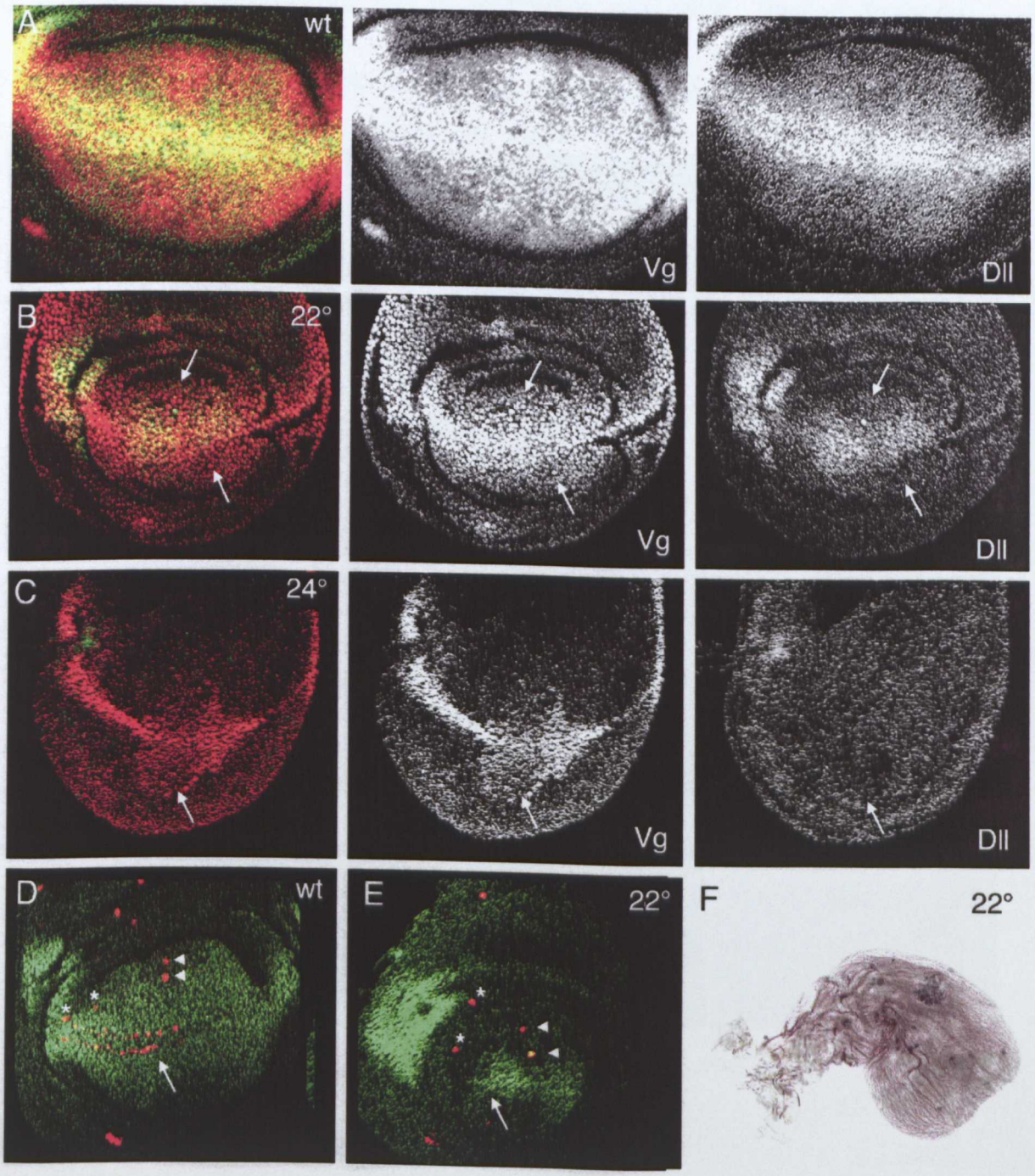

A
wt
Vg
Dll
B
22°
Vg
Dll
C
24°
Vg
Dll
D
wt
E
22°
F
22°

Figure 6.4.2: Cell-autonomous reduction of Vestigial and Distal-less expression in *dsh* mutant clones.

(A) Clone of cells homozygous for dsh^{VA153} (arrow). The clone is marked by the absence of N-myc expression (green in A, single channel below). Note the reduction of Vg expression (red) within the clone.
(B) Clone of cells homozygous for dsh^{75}. The clone is marked as in A. Note that reduction of Vg within the clone is strongest in the cells of the clone that are far away from the DV boundary. The arrowheads indicate the DV boundary.
(C) Clone of cells homozygous for dsh^{VA153} . The clone is marked as in A. Note the reduction of *Dll-lacZ* expression (red) in the clone and the narrowness of the expression domain. The arrowheads indicate the DV boundary.

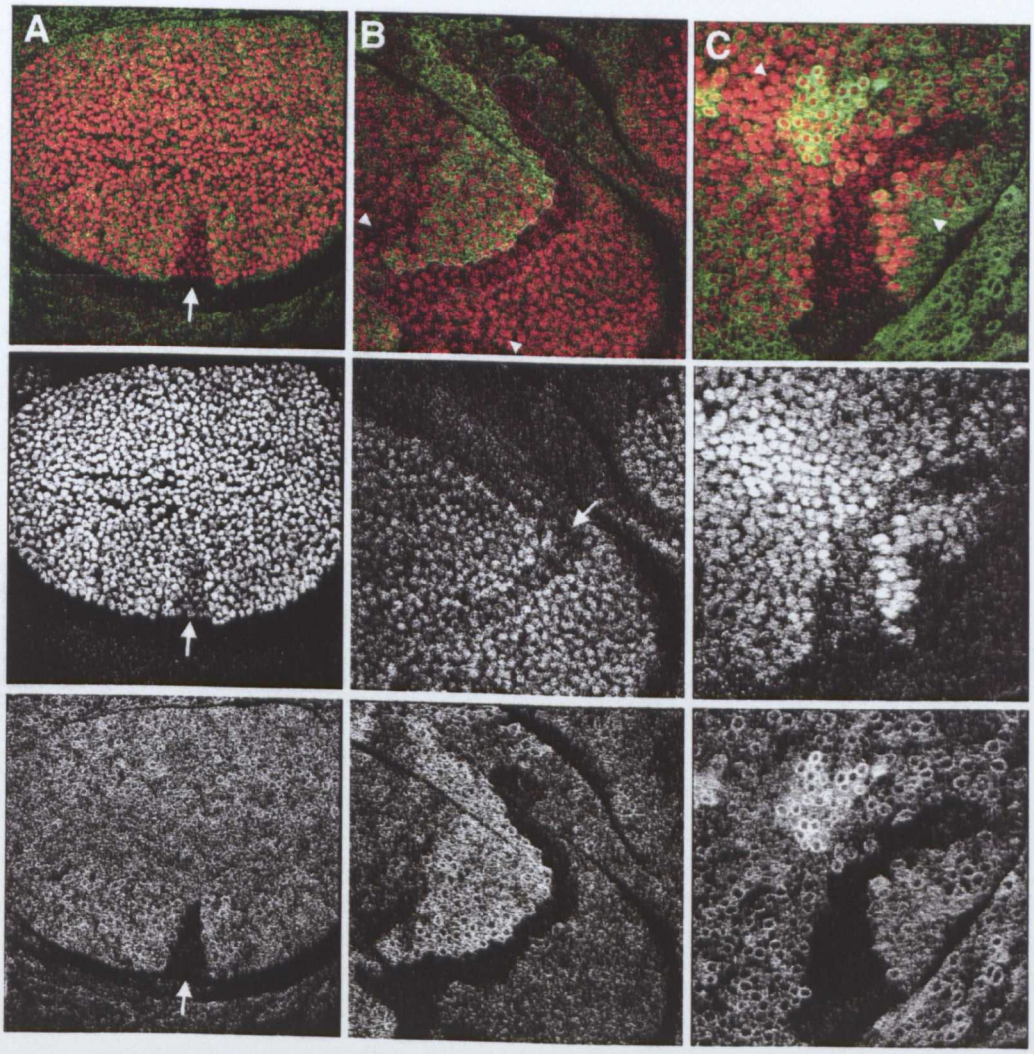
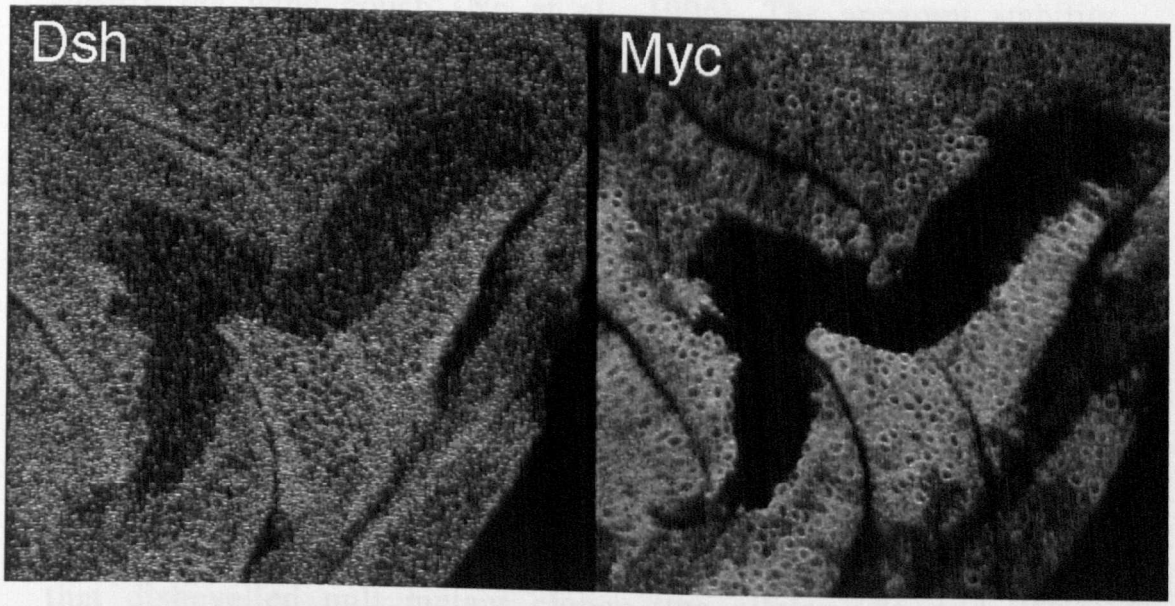

A
B
C

Figure 6.4.3: *dishevelled* null mutant clones show reduced, but not eliminated staining for Dishevelled

Large dsh^{v26} clone, induced in first instar and marked by the absence of *myc* staining (right panel) and also stained for Dsh protein (left panel). Note that although the level of Dsh protein is reduced inside the clone, a low level of staining appears to persist.


Dsh
Myc

The observation that Distal-less and Vestigial expression are reduced more strongly in *dishevelled* mutant cells at a distance from the source of Wg, than in cells near the source, suggests that the level of Wg activity is graded across the wing pouch. The *dishevelled* alleles used here (VA153, 75, and v26) have been characterized genetically and molecularly as null alleles (Perrimon and Mahowald, 1987; Klingensmith et al., 1994). Larvae homozygous for *dishevelled*^{v26} (which has been shown to be a protein null mutant; see Klingensmith et al., 1994), develop wing discs with a discrete wing pouch despite the absence of zygotic *dishevelled* activity (Couso et al., 1994). This indicates that the maternal *dishevelled* product persists at least until the mid-second larval instar when Wg is required to specify the wing pouch (Ng et al., 1996). The apparent stability of the *dishevelled* product raises the possibility that *dishevelled* null mutant clones retain some Dishevelled activity. Clones of cells mutant for the *dishevelled* null alleles show reduced, but detectable, levels of Dishevelled protein (Fig. 6.4.3), suggesting that these clones behave as though Dishevelled activity were reduced, but not completely removed, which may explain why Distal-less and Vestigial expression is reduced most strongly at a distance from the wing margin, where Wg activity should be lowest, but not in regions where Wg activity is high.

Couso et al. (1994) and Rulifson et al. (1996) have shown that dishevelled null mutant clones (the alleles v26 and 75) autonomously lose bristle cell fates and Acheate-scute expression at the wing margin. This suggests that a partial reduction of the activity of the Wg pathway in *dishevelled*$^-$ clones is sufficient to cause complete loss of Acheate-scute expression, while Distal-less and Vestigial are only reduced. This is consistent with the proposal that a very high level of Wg signal is required to activate *acheate-scute* expression, while lower levels are sufficient to activate *Distal-less* and *vestigial* (although expression is weakened in *dishevelled*$^-$ clones).

6.5: The level of Wg signalling controls growth and the specification of cell fate along the D/V axis of the wing

Data presented in chapter 3 above indicate that *spdfg* and *Sp* are regulatory mutations that reduce Wg expression at the D/V boundary of the wing disc. It is possible to order various *wg* allelic combinations with these mutations into an allelic series (Fig. 6.5.1), ranging from a weak reduction of Wg expression at the D/V boundary in *spdfg* homozygotes to a very strong reduction in the allelic combination $wg^{CX4}/spdfg$ *Sp*. The stronger the reduction of Wg expression at the D/V boundary, the smaller the resulting wing becomes (Fig. 6.5.1), suggesting that the level of Wg signalling determines the size of the wing blade. Reducing the level of Wg signalling reduces both the maximum level of Distal-less expression near the D/V boundary and the distance from the D/V boundary at which Distal-less can be activated (Figs. 6.5.1 and 6.5.2). Note the proximity of the Distal-less expressing sense organs to the D/V boundary in the mutant disc (arrows, Fig. 6.5.2F). In wild-type discs these cells are normally found near the edge of the wing pouch at a considerable distance from the D/V boundary (arrows, Fig. 6.5.2E). These observations suggest that reducing Wg activity leads to loss of cell fates that normally occur close to the D/V boundary. Cell types normally found at a distance from the boundary, where Wg activity is expected to be low, are still specified in the mutant disc, but in a position much closer to the D/V boundary.

Figure 6.5.1: *wg* mutants affecting the wing can be ordered in an allelic series

(A - D) adult wings. (E - H) Wingless protein. (I - L) Vestigial protein.. (M - P) Distal-less protein. (A, E, I, M) wild-type. (B, F, J, N) *spdfg* homozygous. (C, G, K, O) *spdfg*/Df*spd*^{hL2}. (D, H, L, P) *spdfg Sp/wg*^{CX4}.
Note that in the progressively stronger allelic combinations, more and more wing blade tissue is lost. This correlates with a progressively lower level of Wg expression, and a concomitant reduction, both in intensity and in size, of the Vg and Dll expression domains.

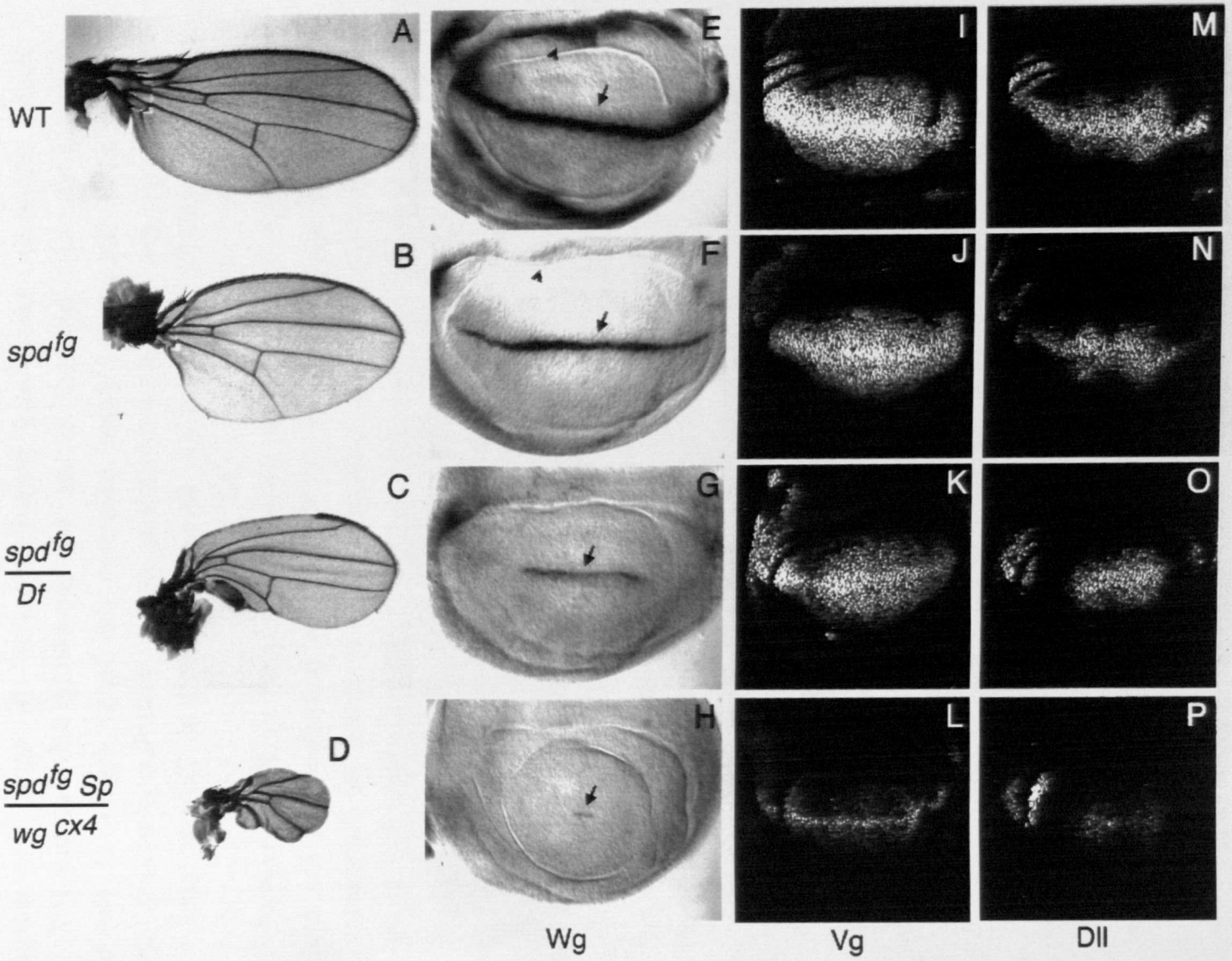
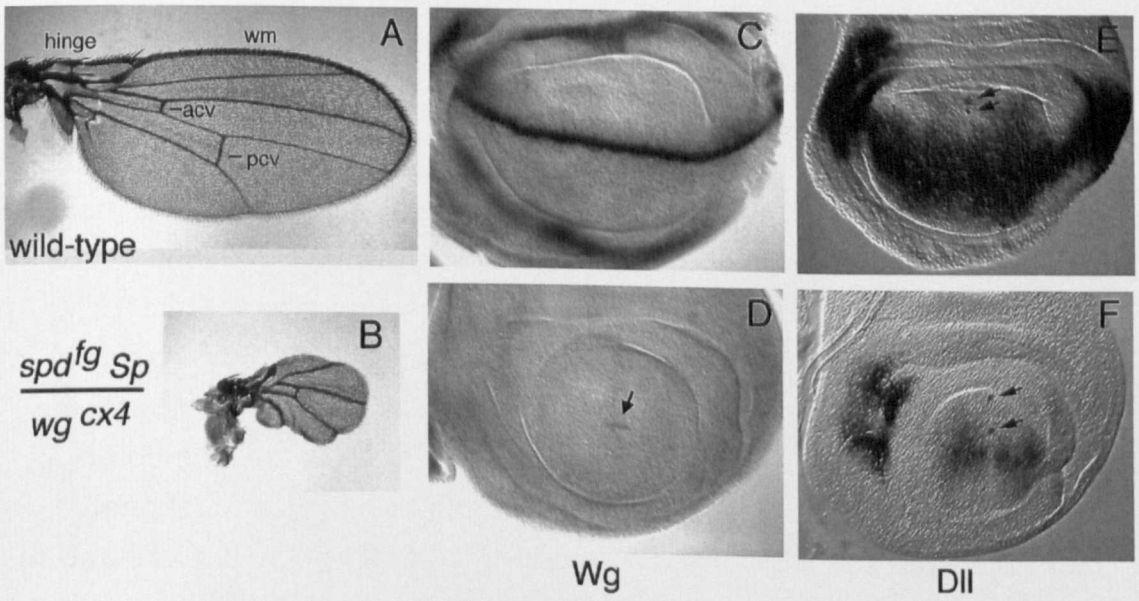

WT
A
E
I
M
B
spd fg
F
J
N
C
spd fg
Df
G
K
O
D
spd fg Sp
wg cx4
H
L
P
Wg
Vg
DII

Figure 6.5.2: Loss of wing tissue and alteration of target gene expression in *wg* mutant discs

(A) wild-type adult wing. The wing margin (wm) consists of rows of bristles along the dorsal and ventral edges of the wing. The margin is produced by cells adjacent to the Wg-expressing cells at the DV compartment boundary. Hinge indicates the hinge region at the base of the wing. acv = anterior crossvein, pcv = posterior crossvein. (B) *spdfg* Sp/wg^{cx4} mutant wing. Structures distal to the pcv are missing.
(C) Wg protein expression in wild-type and (D) *spdfg* Sp/wg^{cx4} mutant wing discs. The level of Wg protein is reduced in the mutant, and can only be seen as a faint patch of expression at the center (arrow).
(E) Distal-less protein expression in wild-type and (F) *spdfg* Sp/wg^{cx4} mutant discs. In wild-type Dll expression is highest at the D/V boundary and decreases in intensity away from the wing margin. The arrows in panels (E, F) indicate sense organs near the base of the wing pouch that label with Dll antibody. (F) In the mutant disc Dll is expressed at a much lower level at the D/V boundary and in a much narrower domain. Note the proximity of the distal-most sense organ to the DV boundary. Note also that the separation between the sense organs is increased. This could be due to a shallower gradient of Wg protein (and hence positional information) emanating from the DV boundary in the mutant disc.


hinge
wm
A
acv
pcv
wild-type
C
E
spd^fg Sp
wg cx4
B
D
F
Wg
Dll

6.6 The mature expression of *vestigial* and *Distal-less* is gradually established during wing development

The Wg-dependent expression of *vestigial* and *Distal-less* in the wing pouch is initiated in early third instar (Fig. 6.6.1). At this early stage, only a few cells that are close to the D/V boundary express Vestigial and Distal-less, and the level of expression of these two genes is weaker than that found in mature discs (Fig. 6.6.1). As the disc develops, the expression domains of Vestigial and Distal-less get broader and stronger. This suggests that the level of Wg signalling in the early wing pouch may not be as strong as in the mature disc. Consistent with this psoposal, the level of Wg antigen at the D/V boundary of early third instar wing discs is significantly weaker than that of late third instar discs (data not shown). Also consistent with this proposal, Vestigial expression appears to come on before Distal-less expression in early third instar (data not shown), followed by the activation of Acheate-Scute expression not until mid-third instar, about 20 hours later (Blair, 1992). Interestingly, Acheate-Scute expression is initiated at roughly the same time as Cut expression at the wing margin (Blair, 1993), which correlates well with the observation that both genes appear to be equally sensitive to the Wg signal (Rulifson et al., 1996; Fig. 5.3.1D). The expression of both genes is lost in *wg* mutant clones at the margin, and is only rescued in cells that are immediately adjacent to Wg-expressing cells.

Figure 6.6.1: The temporal dynamics of Vestigial and Distal-less expression in the wing disc

(A - D) Late second instar/early third instar wing discs arranged with the youngest disc at the left (A) and progressively older stages towards the right. All discs are labelled for Vestigial protein (red) and Distal-less protein (green). Discs were stained and photogtaphed together. Note that Dll expression is activated at very low levels in the center of the disc (A, B), and that expression becomes progressively stronger in older discs, and spreads away from the D/V boundary, and to the lateral regions of the disc (C, D). Vestigial expression follows the same sequence, but appears to be activated in the wing pouch (*ie.* in response to Wg) slightly earlier than Distal-less.
Note also that Dll is activated first in the center of the disc. This correlates well with the observation that the mature Dll expression domain is broadest in the central part of the wing pouch (eg. see Fig. 6.4.1A). This is likely to be due to a sensitization of these cells to Wg by Dpp (Kim et al., 1996; data no shown).

A
B
C
D
Vg
Dll

These observations suggest that the Wg gradient of positional information is gradually developed during the 48 hours of development between late second instar and late third instar, and that the responding cells continuously monitor their position in this gradient. Since cells that initially respond to a low level of Wg appear to be able to switch their response later on to a high level of Wg, their final state of commitment must remain plastic until late in development. Similar observations were made by Gurdon et al. (1995), who found that cells initially exposed to a low level of Activin could switch their response if subsequently exposed to a high level of Activin.

6.7: Discussion

6.7.1: Wg acts directly on all cells of the developing wing blade

Ectopic expression of Wg in the developing wing blade can induce formation of an ectopic wing margin as well as outgrowths from either the dorsal or ventral surface of the wing (Diaz-benjumea and Cohen, 1995; Fig. 6.3.3). Specification of wing margin fate occurs in cells immediately adjacent to the source of Wg signal, reflecting a requirement for high levels of Wg activity (Blair 1992; 1994; Phillips and Whittle, 1993; Couso et al., 1994). The long-range effect of Wg-expressing clones on growth of non-adjacent cells suggests that Wg can also act at a distance to influence cell behaviour. I have presented data in this chapter that Wg, expressed at the D/V boundary, acts over long distances to control the expression of *Distal-less* and *vestigial* in the wing pouch. One can consider two models for how Wg might act at a distance to direct target gene expression. Wg protein might act directly to induce expression of its target genes in cells distant from the source.

Alternatively, Wg might act locally to induce a second signal that relays information to more distant cells.

Two lines of evidence argue against a signal relay mechanism for Wg. First, I have shown here that clones of cells reduced in their ability to transduce the Wg signal lose expression of Distal-less and Vestigial. This indicates that the ability to receive the Wg signal is required directly by all cells of the wing pouch, independent of their position. Second, if there were a signal relay mechanism one would expect cells in which the Wg signal transduction pathway is activated to exert a non-autonomous influence on surrounding cells. This has been examined in two ways. First, removing activity of the *shaggy/zeste-white3* kinase is equivalent to activating the Wg pathway (Siegfried et al., 1992). Cells mutant for *shaggy/zeste-white3* in the wing blade express three different targets for Wg signalling: the proneural proteins of the Acheate-Scute complex, Distal-less and Vestigial (Blair, 1992; 1994; Diaz-Benjumea and Cohen, 1995). Activation of these target genes is strictly autonomous to *shaggy/zeste-white3* mutant cells and is not seen in surrounding wild-type cells. Furthermore, although *shaggy/zeste-white3* mutant cells differentiate as wing margin cells they do not exert a non-autonomous influence on surrounding cells, in contrast to Wg-expressing cells, which do both (Diaz-Benjumea and Cohen, 1995). Second, the Wg pathway can also be activated by over-expressing *dishevelled* (Yanagawa et al., 1995; Axelrod et al., 1996; Neumann and Cohen, 1996a). Over-expressing *dishevelled* using the GAL4 system leads to ectopic specification of wing margin bristles (Axelrod et al., 1996; Neumann and Cohen, 1996a), and to cell autonomous activation of Distal-less and Vestigial (Fig. 6.3.3B,C). Also, Zecca et al. (1996) have shown that expressing a membrane-tethered form of Wg only has a short-range effect on target gene expression, in contrast to secreted Wg. Taken together, these observations effectively rule out the possibility that the long-range action of Wg might be mediated by relay of another downstream signal and

strongly suggest that Wg acts directly on all cells of the wing pouch.

6.7.2: Multiple thresholds for Wg activity along the D/V axis of the wing

Wg acts non-autonomously to define the expression domains of at least 3 different target genes in the wing pouch: *acheate-scute*, *Distal-less* and *vestigial*. Because Acheate-Scute expression is restricted to cells in very close proximity to the source of Wg expression at the D/V boundary this can be considered to reflect a requirement for high levels of Wg activity. Distal-less is expressed in a much broader domain and in a graded manner with high levels centered on the domain of Wg expression. Vestigial is expressed throughout the entire wing pouch. In all three cases Wg appears to act directly to control expression, because activity of the *wg* signal transduction pathway is directly required for target gene expression in responding cells, even at late stages of development, and (in the case of *Distal-less* and *vestigial*) at a distance from the D/V boundary. This suggests that different thresholds of Wg activity are required to define the spatial domains of expression of these three target genes (Fig. 6.7.1). Consistent with this proposal, reducing the level of Wg activity at the D/V boundary to an intermediate level leads first to the loss of wing margin cell fates, while still allowing the activation of Distal-less and Vestigial (Fig. 6.4.1B,E,F, although the domains of Distal-less and Vestigial become narrower and weaker). A further reduction of Wg activity leads to the loss of Distal-less expression, while Vestigial expression is still activated at low levels (Fig. 6.4.1C). It should be noted that Distal-less is expressed at low levels in cells near the edge of the Vestigial domain, so the difference between the minimal level of Wg activity required to activate Distal-less and Vestigial is not very different. Consistent with the data

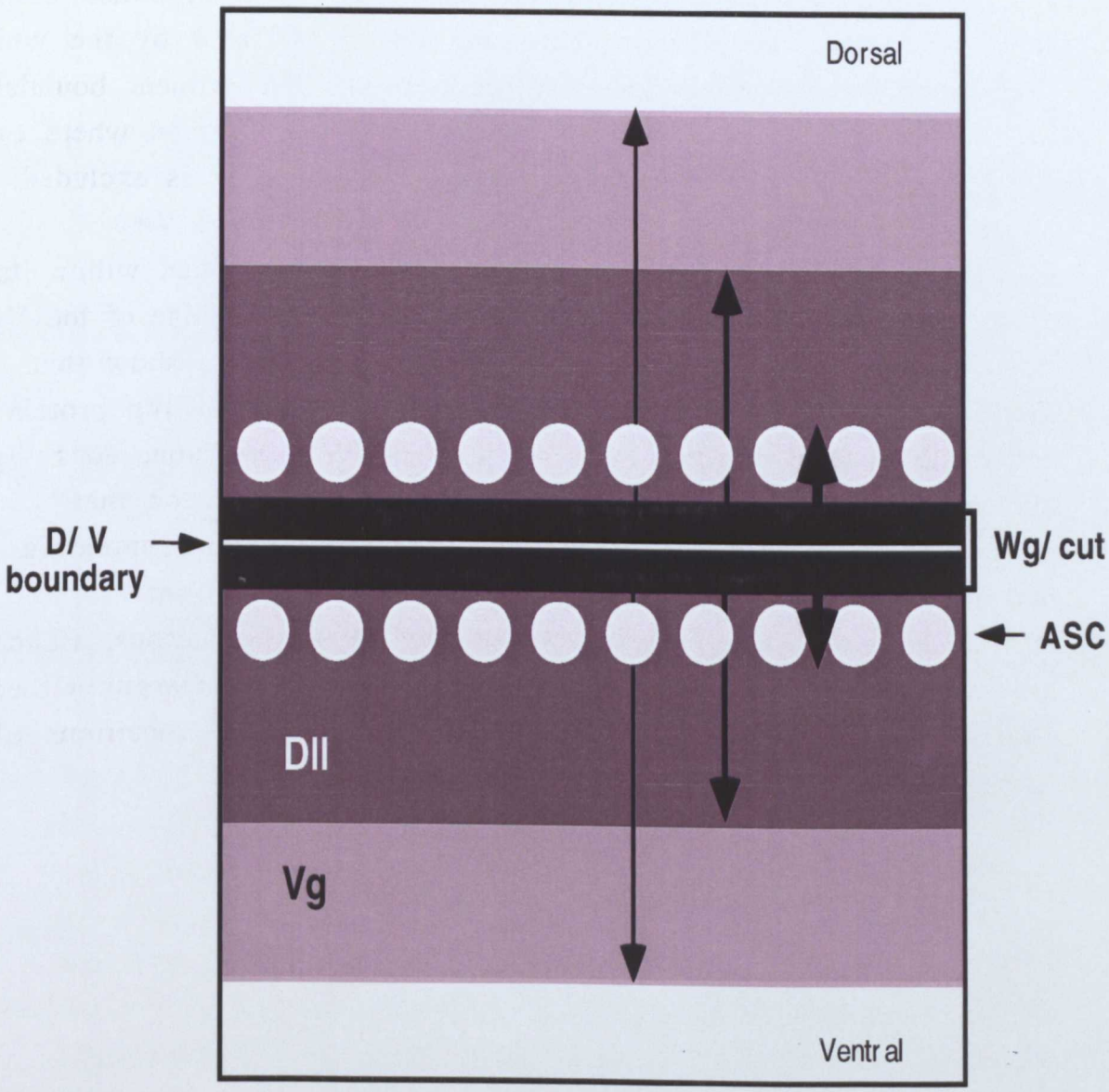
described here, Zecca et al. (1996) have shown that expressing Wg at relatively low levels is sufficient to activate Distal-less expression, but not Acheate-Scute expression.

Wg is also required in conjunction with Notch to define another domain: the narrow band of cells between the rows of sense organs at the wing margin. Cut is expressed in these cells and depends on both Wg and Notch activity (Couso et al., 1994; Neumann and cohen, 1996b; see also Chapter 5 of this thesis). Taken together, these results indicate that distinct activity thresholds can be defined by Wg protein, and by Wg protein in conjunction with Notch signalling, leading to the generation of several different cell states along the D/V axis of the wing (Figs. 5.4.1 and 6.7.1).

An important question raised by these results is how Wg regulates growth in the wing pouch. In this context, it is interesting to note that a very strong activation of the wg pathway in *shaggy/zeste-white3* mutant clones does not lead to overgrowth within the clone. Likewise, ubiquitous strong expression of Wg or Dsh does not lead to overgrowth in the wing pouch, while it does so in the wing hinge (Fig. 4.3.1). However, expressing high levels of Wg from a localized source does cause overgrowth of surrounding tissue (Diaz-Benjumea and Cohen, 1995; Fig. 6.3.3). This suggests that only an intermediate level of activation of the wg pathway can stimulate growth in the wing pouch. This would correspond to the cells which receive enough Wg to activate Distal-less and Vestigial, but not enough to activate the Acheate-Scute complex. Phillips and Whittle (1993) have shown that a zone of non-proliferating cells at the D/V boundary depends on *wg* activity for its quiescence. This suggests that, paradoxically, Wg stimulates growth in the wing pouch at low concentrations, while inhibiting it at high concentrations.

Figure 6.7.1: Model for long-range organizing activity of Wg in the developing wing.

The wing disc is depicted by a box. The disc is divided into dorsal and ventral compartments by expression of Apterous in dorsal cells (not indicated). The compartment boundary is indicated by the white line. Wg is expressed by cells adjacent to the compartment boundary in both compartments (black band). This is also the region where *cut* is expressed and from which expression of AS-C genes is excluded. Dll expression is depicted by medium gray shading and Vg expression by light gray shading. Dll expression is graded within its domain, and low levels of Dll can be seen close to the edge of the Vg domain. For simplicity the drawing does not attempt to show this. We propose that the local concentration of the secreted Wg protein defines the spatial domains of AS-C, Dll and Vg expression. Thus Wg may act as a morphogen to provide positional information that patterns the DV axis of the wing. The effects of Wg are symmetric, but dorsal cells are programmed to interpret the positional information differently, because of the presence of Apterous. Hence the dorsal wing margin differs from the ventral wing margin. Other differences in dorsal and ventral compartments include positions of sense organs.


Dorsal
D/ V
boundary
Wg/ cut
ASC
DII
Vg
Ventral

6.7.3: How is the Wg gradient generated and how is it read?

How does the Wg protein travel across the wing pouch from the Wg-secreting cells at the D/V boundary? One possibility is by passive diffusion through the extracellular matrix. This is supported by the observation that soluble, biologically active Wg protein can be recovered from the medium of Wg-secreting cells (van Leeuwen et al., 1994). Also, Gurdon et al. (1994) have shown that Activin can travel across a layer of cells in which protein synthesis has been inhibited, and signal to cells on the other side, suggesting that it may travel by passive diffusion.

Arguing against passive diffusion of Wg is the observation that Wnts appear to adhere very tighly to the extracellular matrix (Papkoff & Schryver, 1990) and that Wg protein found outside of Wg-secreting cells is always observed in vesicular structures inside nearby responding cells (van den Heuvel et al., 1989; González et al., 1991; Fig. 6.6.2). These results raise the intriguing possibility that Wg protein may be actively transported away from its source by responding cells. Bejsovec & Wieschaus (1995) have presented data in favour of this proposal. In embryos, Wg protein can be found in vesicular stuctures of responding cells up to three cells away from its source, as can the accumulation of Armadillo protein, which is a response to the Wg signal (Peifer et al., 1994). The *shibire* gene encodes a dynamin homologue that is essential for endocytosis (Chen et al., 1991). Bejsovec and Wieschaus use a *shibire*^{ts} mutant to show that abolishing endocytosis does not abolish the ability of cells to receive the Wg signal, as Armadillo is still stabilized in cells close to the Wg-secreting cells, suggesting that the Wg signal is transduced at the cell surface. However, only the Wg-expressing cells themselves, and the cells immediately adjacent to them respond to Wg in *shibire* mutant embryos, suggesting that the range of Wg signalling is reduced under these conditions, and that

endocytosis of the Wg protein by responding cells may be necessary to propagate the Wg signal to cells that are not in direct contact with Wg-expressing cells. It is necessary to interpret this result with caution, however, as the reduction of *shibire* function impairs the viability of cells, and thus may be indirectly affecting Wg signalling.

The model proposed here for long-range signalling by Wg in the wing pouch (Fig. 6.7.1) bears a strong resemblance to the Local Source Dispersed Sink (LSDS) model (reviewed in Slack, 1987, 1991). This model requires not only that the signal diffuses away from a localized source, but also that it is degraded by responding cells, thereby leading to the formation of a concentration gradient. If Wg travels by passive diffusion, it is possible that it is taken up and degraded by the responding cells, which could account for the Wg protein found in vesicular bodies inside responding cells. If Wg is actively transported, cells may only pass on part of the Wg protein that they take up.

Other important questions that remain to be answered are how cells measure the Wg gradient, how this gradient elicits different responses, and how it controls growth.

The weakest aspect of the gradient model of positional information has always been its interpretation by responding cells (Wolpert, 1989). It is difficult to imagine how the graded expression of a single molecule can be translated into a very precise arrangement of specific pattern elements, as well as how it can regulate the size and proportions of a field of cells. In this context, it is interesting to compare Wg with several other putative morphogens. Activin has been proposed to function as a morphogen in the amphibian blastula (Green and Smith, 1990; Green et al., 1992; Gurdon et al., 1994, 1995). These studies show that Activin can specify several different cell types in a concentration-dependent manner. However, other studies (Green et al., 1994; Symes et al., 1994; Wilson and Melton, 1994) have presented data suggesting that this effect may be rather indirect. The initial response to the

Activin gradient appears to be a relatively crude prepattern that is characterized by continuous cell states. Subsequent interactions among the responding cells elaborate this pattern and generate thresholds, thereby giving rise to the distinct cell types found at different Activin concentrations. These data suggest that a morphogen gradient can affect pattern formation indirectly, by initiating a cascade of further cell-cell interactions. This may be a general rule, as the Drosophila Bicoid morphogen induces the expression of *hunchback*, which itself functions as a morphogen (Struhl et al., 1992), and these two genes synergistically initiate a whole cascade of gap, pair-rule, and segment polarity genes that control patterning of the embryo (reviewed in St. Johnston and Nuesslein-Volhard, 1992). Likewise, the Dorsal morphogen gradient leads to the expression of *dpp* dorsally and *sog* ventrolaterally, and interactions between these secreted factors elaborates pattern formation in the D/V axis of the embryo (Biehs et al., 1996). Dpp itself has been proposed to function as a morphogen in this context (Ferguson and Anderson, 1992), and also functions as a morphogen in the wing pouch (Nellen et al., 1996; Lecuit et al., 1996). In the wing pouch, Dpp specifies the location of veins in the A/P axis (Zecca et al., 1995; DeCelis et al., 1996b; Sturtevant et al., 1997). Once again, this appears to be a rather indirect effect, as veins are initially specified as 'vein-competent' areas, which are subsequently refined by local interactions involving Dpp and EGF-receptor signalling (pro-vein) and lateral inhibition by the Notch pathway (anti-vein; Garcia-Bellido and DeCelis, 1992; Sturtevant & Bier, 1995; Yu et al., 1996; Sturtevant et al., 1997).

Compared to these read-outs, the responses of Wg target genes appear to be fairly direct in the wing pouch. However, it is necessary to ask how the activity of these target genes relates to the final pattern elements formed in response to Wg.

The Acheate-Scute genes are necessary for the formation of innervated bristles at the wing margin (Skeath & Carroll, 1991). However, not every cell that activates Acheate-Scute

transcription in response to Wg goes on to become a bristle mother cell. Acheate-Scute expression is initially activated in two stripes flanking the Wg-expressing cells, followed by a restriction of expression to an evenly spaced subset of this population, which then become bristle mother cells (Blair, 1992). This restriction of expression is due to lateral inhibition mediated by the Notch pathway (Rulifson and Blair, 1995). Thus the specification of bristle cell fate by Wg appears to be relatively indirect, due to the generation of a 'bristle-competent' region analogous to the 'vein-competent' regions specified by Dpp, which is then further patterned by local cell interactions.

Distal-less appears to have only a minor role in wing development, being only required for some aspects of bristle differentiation at the wing margin (F. Diaz-Benjumea, personal communication). Thus its Wg-dependent expression in the wing only serves as a marker for the gradedness of Wg signalling across the wing pouch.

As discussed above (section 5.5.2), the function of Vestigial is still rather mysterious. It has been proposed that Vestigial is required for growth of the wing, and so it is tempting to speculate that Wg controls growth through the activation of Vestigial expression in the wing pouch. However, this model is certainly much too simple. Thus there is no indication that the level of Vestigial expression has any effect on proliferation (S. Carrol, personal communication). If this is not the case, how can the level of Wg expression at the D/V boundary control the size of the wing? Also, Vestigial is already expressed throughout the entire wild-type wing pouch, and yet it is possible to induce overgrowth of these cells by expressing Wg ectopically in the wing pouch. Taken together, these results suggest that while Vestigial expression in the wing pouch in response to Wg is essential, it does not regulate growth.

If not through Vestigial, how does Wg control growth of the wing pouch? As noted above, a very strong ubiquitous

activation of the Wg pathway does not cause overgrowth of the wing pouch, while the expression of Wg from a localized source does. One way to interpret this is that it is the gradient of Wg activity which controls proliferation (and/or cell death). It has been proposed that a morphogen gradient can impart three different types of information on responding cells (reviewed in Wolpert, 1969, 1989; Lawrence, 1992; Lawrence and Struhl, 1996). The scalar is the concentration of the morphogen molecule at any one point, and provides a measure of distance from the source of the morphogen, the D/V boundary in this case. At the same time, the vector of the concentration gradient could impart polarity on the responding cells, and the slope could control the size of the field. Data presented in section 6.6 above suggest that the level of Wg expression at the D/V boundary is initially weak, and becomes gradually stronger during development. If the Wg protein spreads rather slowly from its source, this could lead to a steepening of the slope in the responding cells, which could then trigger cell division. However, it remains difficult to imagine how cells could accurately measure the steepness of the Wg slope, although one possibility could be through the regulation of expression of an adhesion molecule, perhaps expressed in a gradient similar to that of Distal-less (see Fig. 6.4.1A).

An alternative way in which Wg could control growth in the wing pouch is by the activation of local mitogens at specific threshold levels. Data presented in chapter 4 above suggest that Wg itself functions as a local mitogen in the hinge region of the wing, and that proliferation in this part of the disc can be abolished without affecting proliferation globally. Also, localized expression of Wg in the hinge is probably directed by a putative hinge organizing center (Ng et al., 1995; Neumann and Cohen, 1996a), suggesting that other organizing centers may similarly activate local mitogens. In this context, it is interesting to note that the gene *four-jointed* is required specifically for the generation of the cell population located between the two cross-veins of the Drosophila wing (Villano

and Katz, 1995). In the absence of *four-jointed* function, the cross-veins are moved closer together, thus leading to a shortening of the wing, and the authors suggest that this effect is not due to increased cell death, implying that it is instead due to locally reduced proliferation. Thus it may be that a number of different genes control proliferation in small domains of the disc, and that these genes are in turn regulated by the global positional information generated by the organizing centers.

CHAPTER 7:
CONCLUSIONS

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In this thesis, I have presented data suggesting that *spdfg* and *Sp* are regulatory mutations of *wingless*, each affecting a different subset of larval functions. *spdfg* reveals a mitogenic function of Wingless in the wing hinge. *spdfg* and *Sp* both reduce Wingless expression at the D/V boundary, and both have been useful in determining the precise position of Wingless in the genetic hierarchy that establishes and mediates the activity of the D/V organizer of the wing. *wingless* and *vestigial* are parallel targets of the Notch pathway at the D/V boundary. Wingless and Notch subsequently cooperate to activate *cut* at the D/V boundary. Wingless also functions as a mediator of both the short- and long-range effects of the D/V organizer. Short-range effects are mediated by the activation of genes of the *acheate-scute* complex, while long-range effects are mediated by the activation of *vestigial*. *Distal-less* is also regulated by Wingless in the wing pouch. Analysis of the regulation of these three target genes suggests that Wingless acts directly at a distance to activate their expression, and that it defines the different expression domains by signalling in a concentration-dependent manner. Taken together, these results suggest that Wingless mediates the activity of the D/V organizer by acting as a morphogen. As Dpp also appears to function as a morphogen in mediating the organizing activity of the A/P organizer (Nellen et al., 1996; Lecuit et al., 1996), this raises the possibility that organizer activity is mediated by morphogens in general.

7.1: Future Directions

Among the more interesting problems that remain to be answered is the question of how a morphogenetic gradient like that of Wingless is read by responding cells, how this is translated into specific pattern elements, and how it is used to

control size and proportions. There appears to be no easy way to address this problem with the currently availabe tools. Perhaps the identification of additional targets of Wingless signalling could shed more light onto this question.

Another interesting question is whether the Wingless protein travels by passive diffusion in the extracellular space. Gurdon et al. (1994) have shown that Activin can cross a layer of cells in which protein synthesis is inhibited. It may be possible to generate clones of a temperature-sensitive cell-lethal mutation in the wing disc, and then ask if Wingless protein is able to coss a patch of dying or dead cells and activate target gene expression on the other side (in other words, whether the dead cells would cast a 'shadow' on the wild-type cells on the far side).

One would also like to know the position of Wingless in the genetic hierarchy which is responsible for organizing the hinge region of the wing. This represents a patterning event in the proximal/distal (P/D) axis of the wing, and there are data which suggest that this may be under the control of a distinct P/D organizing center, which may be located in the proximal hinge (Ng et al., 1995). Interestingly, this location corresponds to a compartment boundary (Garcia-Bellido et al., 1973).

Finally, it would also be interesting to know which factors are responsible for controlling the competence of different cells (*eg.* cells in the hinge versus the wing pouch) to respond differently to a signal like Wingless. The expectation is that specific transcription factors, analogous to selector genes, might fulfill this function, and these should emerge out of the analysis of the long list of mutations known to affect imaginal disc development.

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